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Mechanical stress to *Drosophila* larvae stimulates a cellular immune response through the JAK/STAT signaling pathway

Yumiko Tokusumi [1], Tsuyoshi Tokusumi [1], Robert A. Schulz*

Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA

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ABSTRACT

Acute inflammation can cause serious tissue damage and disease in physiologically-challenged organisms. The precise mechanisms leading to these detrimental effects remain to be determined. In this study, we utilize a reproducible means to induce cellular immune activity in *Drosophila* larvae in response to mechanical stress. That is, forceps squeeze-administered stress induces lamellocytes, a defensive hemocyte type that normally appears in response to wasp infestation of larvae. The posterior signaling center (PSC) is a cellular microenvironment in the larval hematopoietic lymph gland that is vital for lamellocyte induction upon parasitoid attack. However, we found the PSC was not required for mechanical stress-induced lamellocyte production. In addition, we observed that mechanical injury caused a systemic expression of Unpaired3. This cytokine is both necessary and sufficient to activate the cellular immune response to the imposed stress. These findings provide new insights into the communication between injured tissues and immune system induction, using stress-challenged *Drosophila* larvae as a tractable model system.

1. Introduction

The inflammatory response is a key initial defense to host physiological or environmental challenge. It is one of the first lines of defense involving humoral and cellular immune activity to combat a potential threat to an organism. However, the response is potentially of multiple differing effects as it may contribute positively in host defense such as against invading microbes, but may also culminate in undesired damage to host tissue(s). An inflammation response can occur not only in an infectious condition, but also due to non-infectious stimuli such as exposure to mechanical stress, ischemia, toxins, minerals, crystals, chemicals, and antigens [1]. Induced sterile inflammation in many of these animal challenges can potentially cause collateral damage in tissues and result in chronic disease. However, the precise mechanisms of sterile inflammation at molecular and cellular levels remain poorly understood. Toward the elucidation of these mechanisms, systematic genetic and molecular studies are warranted using tractable animal systems. *Drosophila* provides an excellent means for such investigations because of its abundant genetic resources and productive technical approaches. Importantly, the *Drosophila* innate immune system is remarkably similar to that used in vertebrates [2]. Additionally, numerous studies have reported mechanical stress models can induce humoral and cellular immune defense responses in the developing fly [3–7].

Three mature blood cell types can be found in *Drosophila*: plasmatocytes, crystal cells, and lamellocytes [8]. Plasmatocytes are small round cells with phagocytic capacity and they constitute the majority of circulating hemocytes. Crystal cells carry prophenol oxidase, which is involved in melanization. Lamellocytes are large flat adherent cells that are rare under normal developmental and physiological conditions. However, under challenge conditions such as wasp parasitization, numerous defensive lamellocytes are induced, wherein they function to encapsulate the foreign invader.

The embryonic head mesoderm gives rise to hemocytes and these blood cells are contributed to the larval hemolymph [8]. A subpopulation of these hemocytes, termed the sessile hemocytes, eventually come to reside under the larval cuticle. Multidendritic class IV neurons secrete a cytokine of the TGF-β class, that being Actvin-β, to attract hemocytes beneath the cuticle with populations functioning as a cellular microenvironment [9,10]. These embryo-derived hemocytes have the potential to differentiate into other cell types as part of a larval immune response. Additionally, the

* Corresponding author. Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA.
E-mail address: rschulz@nd.edu (R.A. Schulz).
[1] These authors contributed equally to this work.

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class IV sensory neurons contribute importantly in the animal's response to parasitic wasp attack [11].

The larval lymph gland also serves as a vital hematopoietic organ, and it is composed of multiple paired lobes. In third instar larvae, the primary lobes of the lymph gland consist of three distinct functional cellular domains: the cortical zone (CZ), the medullary zone (MZ), and the posterior signaling center (PSC) [12]. The CZ is occupied by differentiated blood cells, while the MZ is composed of a heterogeneous population of blood progenitor cells. In contrast, the PSC functions as a hematopoietic stem cell-like niche for the hematopoietic progenitors [13,14]. In addition, the PSC contributes to the cellular immune response against wasp infestation [13]. An EGFR ligand, Spitz, is produced in the PSC in response to wasp infestation and this leads to a strong induction of the defensive lamellocyte blood cell type in both the lymph glands and hemolymph [15]. Larval genotypes that cause an absence of the PSC cellular domain or Spitz expression in lymph glands results in a dramatic reduction in lamellocyte production in both lymph glands and hemolymph.

We have previously described the use of a forceps-squeezing method to induce a specific immune cell type in larvae, that being lamellocytes [7]. In this study, we investigate genetic and molecular events ongoing in larvae subjected to this means of mechanical stress treatment. Findings include lamellocyte induction in response to mechanical stress is independent of the presence and function of the PSC signaling domain. Mechanical stress also evokes a systemic expression of the cytokine Unpaired3 (Upd3), which activates the organism's immune program. Finally, we show that *upd3* function is both necessary and sufficient to induce cellular immune activity in larvae, as a key inflammatory response to this specific means of animal mechanical stress.

2. Materials and methods

2.1. Drosophila strains

w^{1118}, UAS-*mCD8::GFP*, UAS-*gap::GFP*, UAS-GFP, UAS-tdTomato, UAS-*rpr*, *GMR40C05*, $upd2^{\Delta}$, $upd3^{\Delta}$, $upd2^{\Delta}upd3^{\Delta}$, hop^{27}, $Antp^{17}$, $Antp^{25}$, and *ppk-Gal4* were obtained from the Bloomington Stock Center. *TepIV*-Gal4 was obtained from the Kyoto Stock Center. In addition, the following fly lines were used: *Pcol85* [13]; *hhF4f-GFP* [16]; *MSNF9-mCherry* [7]; eater-GFP [17]; UAS-*upd3* [18]; *PromE800*-Gal4 [19]; *upd3*-Gal4 [20]; *Pxn*-Gal4 [21].

2.2. Wasp infestation

Hymenoptera wasp *L. boulardi* strain Lb17 were reared in *Drosophila* w^{1118} larvae [22]. For wasp infestation experiments, at least 10 female wasps were placed in a fly food vial containing second instar larvae for 24 h and then removed from the vial. After wasp exposure, larval cultures were left for two days at room temperature prior to analyses of lymph glands and hemolyph samples.

2.3. Mechanical stress assays

We initially observed an unintentional low level production of lamellocytes after 24 h when we pinched-transferred third instar larvae with forceps from fly food cultures to glass plates, followed by washing larvae with PBS or water. To prevent this background, it was realized we could prevent inadvertent lamellocyte production if we pinched the most posterior larval segment or scooped larvae in their various transfers (Fig. S1). Larvae were placed on wet filter paper in plastic dishes including fly food, prior to performing squeeze stress procedures with forceps. After mechanical stress, larvae were left in the dishes until analysis. We examined several squeezing conditions to optimize maximum lamellocyte production. Basically, more forceps squeezing events produced more lamellocytes. However, greater than five forceps squeeze events caused substantial animal death. Therefore, it was concluded that five times forceps squeeze was the most effective to induce lamellocytes in a surviving larval test cohort.

Next, we determined the optimal larval developmental stage for treatment. No lamellocyte induction could be determined when squeeze-stressing second instar larvae. Thus early-third instar stage (72–76 h old at 25 °C) and mid-third instar larvae (96–100 h old at 25 °C) were tested. Lamellocytes were inducible at both larval stages, but using the mid-third instar larvae proved the most optimal. Considering the larger larvae were easier to test in lymph gland and hemolyph assays, larvae at the mid-third instar developmental stage were used in all mechanical stress and genetic analyses.

Calibrated Von Frey filaments were made using a slightly modified method to that previously described [23]. Briefly, we used the fishing line Berkley Trilene XL smooth casting 6LB and calibrated ~40 mN force with a balance. As with the forceps squeezing procedure, more filament touches to larvae resulted in greater lamellocyte production. However, filament force presentation more than three times reached a lamellocyte production plateau. Thus it was concluded three touches with the filament was sufficient to produce lamellocytes.

For the puncture assay, a Tungsten needle with a pin holder (Fine Science Tools, CA) was used and mid-third instar larvae were poked with the needle three times. All control and mechanical stressed larvae were left on fly food and dissected 24 h after treatment.

2.4. Analysis of stressed larvae

We observed whole larvae with a Zeiss Lumar V12 fluorescent stereomicroscope system. For analyses of larval hemolymph or lymph glands, methods and protocols previously described by our group were used [16,24], with cell and tissue samples evaluated with a Zeiss Axioscope fluorescent microscope. Images of hemolymph and lymph gland samples were captured with a Nikon A1-R laser scanning confocal microscope.

3. Results and discussion

3.1. Forceps squeezing of Drosophila larvae induces a cellular immune response

We previously reported the use of a forceps-squeezing procedure to induce a specific immune cell type in larvae, that being lamellocytes [7]. To better establish the stress protocol, we determined optimal conditions for lamellocyte induction: mid-third instar larvae were removed from fly food cultures, washed with PBS and pinched 5 times with forceps (see Materials and methods; Fig. S1). With this larval treatment, lamellocytes appeared in lymph glands around 8 h after forceps squeeze, with copious numbers of the immune cells detected in the 12–24 h time frame post stress administration (Fig. 1).

Compared to the cellular immune response elicited by larvae exposed to wasp infestation, we observed some clear differences with larvae subjected to mechanical stress. First upon parasitization, sessile hemocytes become activated and migrate into the hemolymph [5,25]. In contrast in mechanical stress-treated larvae, this hemocyte population did not move into the hemolymph but rather increased in number (Fig. S2). Second upon wasp infestation, MZ blood progenitor cells are rapidly depleted due to

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