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EXTRA VIEW

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Tissue communication in a systemic immune response of *Drosophila*

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ABSTRACT

Several signaling pathways, including the JAK/STAT and Toll pathways, are known to activate blood cells (hemocytes) in *Drosophila melanogaster* larvae. They are believed to regulate the immune response against infections by parasitoid wasps, such as *Leptopilina boulardi*, but how these pathways control the hemocytes is not well understood. Here, we discuss the recent discovery that both muscles and fat body take an active part in this response. Parasitoid wasp infection induces Upd2 and Upd3 secretion from hemocytes, leading to JAK/STAT activation mainly in hemocytes and in skeletal muscles. JAK/STAT activation in muscles, but not in hemocytes, is required for an efficient encapsulation of wasp eggs. This suggests that Upd2 and Upd3 are important cytokines, coordinating different tissues for the cellular immune response in *Drosophila*. In the fat body, Toll signaling initiates a systemic response in which hemocytes are mobilized and activated hemocytes (lamellocytes) are generated. However, the contribution of Toll signaling to the defense against wasps is limited, probably because the wasps inject inhibitors that prevent the activation of the Toll pathway. In conclusion, parasite infection induces a systemic response in *Drosophila* larvae involving major organ systems and probably the physiology of the entire organism.

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Introduction

In contrast to vertebrates, which utilize both innate and adaptive immune responses to battle infection, flies are thought to rely entirely on their well-developed innate immune system. Depending on the type of infection, different functions of this immune system are mobilized. For example, microbes induce a humoral immune response, which is mediated by antimicrobial peptides that are secreted by the fat body, hemocytes, or epithelia,[1,2] and controlled by 2 conserved signaling pathways, the Toll and IMD pathways.[3,4] Eggs laid by parasitoid wasps induce a different type of response, triggering hemocytes (blood cells) to attack and enclose the parasite in a melanized capsule. This cellular immune response involves 3 morphologically distinguishable types of hemocytes: plasmatocytes, lamellocytes, and crystal cells. Lamellocytes are specialized cells that are only seen in the context of an infection and they participate in the encapsulation of the parasite. Several signaling pathways are thought to be involved in this encapsulation response. For instance, loss-of-function mutations in the JAK/STAT (Janus kinase/signal transducers and activators of transcription) or Toll pathways reduce the encapsulation response against wasp infection,[5] while artificial activation of the same pathways triggers a spontaneous immune response, causing hemocytes to aggregate in melanized masses, in a way that is reminiscent of the encapsulation response.[6-11] Altogether, these findings suggested that the JAK/STAT and Toll pathways play a positive role in the cellular defense against wasp infection. Recently, the role of these pathways has turned out to be unexpectedly complex. The cellular response is orchestrated in a systemic interaction between hemocytes, fat body and, surprisingly, the somatic musculature, and this interplay is controlled by JAK/STAT and Toll signaling in these tissues.[12,13]

JAK/STAT activation in muscles is important for cellular immunity

In *Drosophila*, JAK/STAT signaling involves at least 3 cytokines, Unpaired 1, 2 and 3 (Upd1-3), the receptor Domeless (Dome), the tyrosine kinase Hopscotch (Hop), and the transcription factor Stat92E.[14-16] In order to study the role of this pathway in the cellular

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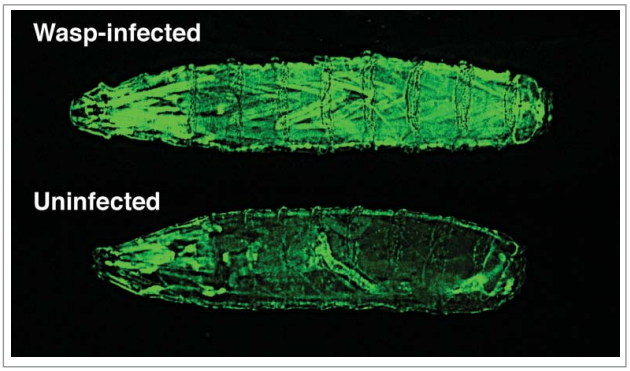

Figure 1. Example of JAK/STAT activation in a *Drosophila* larva, infected by the wasp *Leptopilina boulardi*. Stat92E-dependent transcription is visualized with the *10xStat92E-GFP* reporter.[17] The figure is a montage of 2 photographs, identically exposed and enhanced.

immune response we used the 10xStat-GFP reporter[17] to visualize JAK/STAT activity *in vivo* in wasp-infected *Drosophila* larvae. Surprisingly we observed JAK/STAT activation not only in hemocytes, but also in muscles.[12] This effect, illustrated in Figure 1, was unexpected. So far, research on the cellular immune response in *Drosophila* has focused on what happens in circulating hemocytes and in the haematopoietic tissues of "lymph glands" and sub-epithelial clusters of sessile cells.[15,18,19] Sessile hemocytes have been shown to interact with peripheral neurons,[20] and circulating hemocytes are known to communicate with the fat body as well as with wounds and tumors,[13,21,22] but it was not obvious why the body musculature should also respond to the presence of a parasite egg.

We further tested the role of the JAK/STAT pathway in the immune response by suppressing it separately in those tissues where it was most strongly activated, in plasmatocytes and muscles. It turned out that suppression of JAK/STAT signaling in muscles, but not in hemocytes, significantly reduced the encapsulation of parasite eggs, suggesting that muscles take an active part in the immune response.[12] Occasionally, wasp infection also triggered JAK/STAT activity in a small number of fat body cells but, as shown in Figure 2C, the encapsulation response was not significantly affected when we suppressed JAK/STAT pathway in fat body by expressing the *dome*^{DN} dominant-negative construct with the fat body-specific *FB-Gal4* driver. In conclusion, JAK/STAT-dependent events in the somatic muscles are of particular

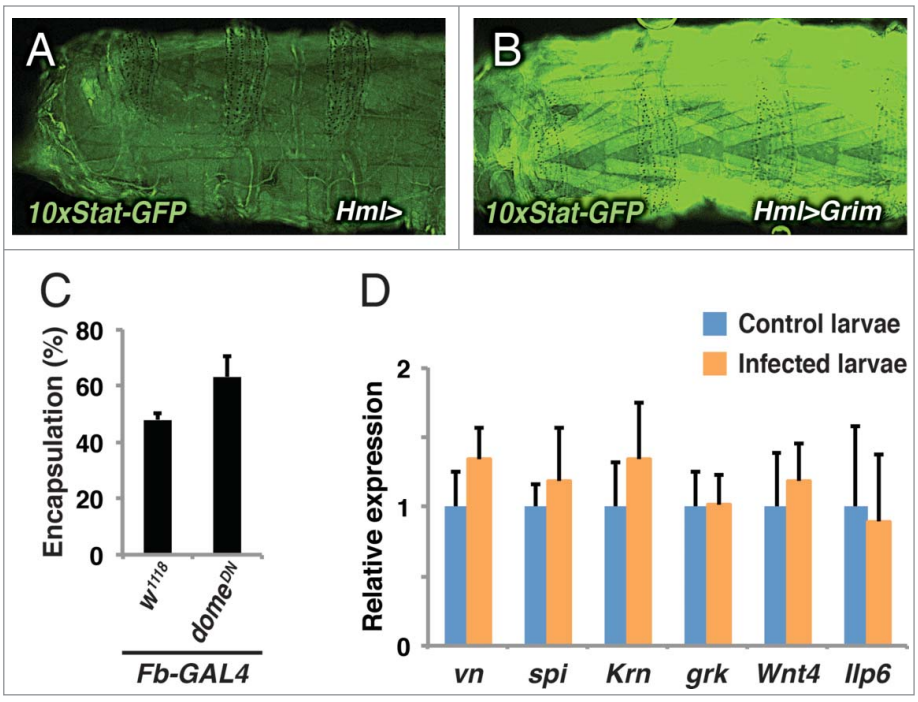

Figure 2. Role of JAK/STAT pathway during cellular immune response (A-B). Compared to the control (A), the JAK/STAT pathway (*10xStat92E-GFP* reporter) is activated in muscles when apoptosis is induced in hemocytes (B). C. The encapsulation of wasp eggs is not affected when JAK/STAT signaling is suppressed in the fat body (*FB-Gal4>dome*^{DN}). Encapsulation rates were scored after 26 hours of wasp infection. Histograms represent average ± standard deviation of at least 3 independent experiments (unpaired t-test). D. The relative expression of the genes for the indicated candidate signaling in skeletal muscles were assayed by quantitative PCR in control larvae (blue bars) and wasp infected larvae (orange bars). Histograms represent average ± standard deviation of at least 4 independent experiments (unpaired t-test).

importance for a successful defense against a parasitoid wasp. Thus, *Drosophila* larval muscles, rather than acting as bystanders, actively participate in the cellular immune response against wasp infection.

Hemocyte signaling to the muscles: The cytokines Upd2 and Upd3

It remains to find out how infection can activate the muscles in the first place and how muscles can affect the cellular immune defense. A partial answer to the first question was found when we traced the origin of the cytokines that stimulate the JAK/STAT pathway. Using reporter strains and quantitative RT-PCR, we found that wasp infection induces the cytokine genes *upd2* and *upd3* in hemocytes.[12] The *upd1* gene is not induced under these conditions, and we saw no induction of *upd2* or *upd3* in other tissues. Furthermore, *upd2* and *upd3* single or double mutants have an impaired cellular immune response against wasp infection. Conversely, artificial overexpression of *upd2* or *upd3* in hemocytes triggers JAK/STAT activity in muscles, causing hemocytes to differentiate into activated lamellocytes.[12] In conclusion, the hemocytes themselves send a signal to the muscles, via the cytokines Upd2 and Upd3, and the response of the muscles is in turn required for hemocytes to efficiently attack the parasite.

The Unpaired cytokines have also been implicated as stress signals under several other circumstances, such as bacterial infection, tumor response, wounding, and virus infection. For instance, bacteremia induces Upd3 production in hemocytes of the adult fly, which activates JAK/STAT signaling in the fat body.[23] This induces the *Turandot A* gene, which encodes a secreted stress peptide of uncertain function.[24] In this case, the response depends on the simultaneous activation of the transcription factor Relish in the fat body.[23] Furthermore, bacterial infection in the gut induces the *unpaired* genes, particularly *upd3*, in intestinal cells. This promotes epithelial regeneration in the gut, which is essential for defense against bacterial infection.[25] As discussed below, this effect is mediated via JAK/STAT activation in the nearby visceral muscles, which respond by secreting the growth factor Vein.[26] A third example is the response to tumors or wounds, which triggers JNK activity and induces Upd1, Upd2, and Upd3 production in local tissues. These cytokines activate the JAK/STAT pathway both in hemocytes and in fat body, which in turn help to restrict tumor growth or defend against infection following wounding.[21] Finally, *Drosophila* C virus or cricket paralysis virus infection induce Upd2 and Upd3 production, and the JAK/STAT pathway contributes to the protection against these viruses.[27]

These examples suggest that Upd2 and Upd3 may be specialized coordinators of a systemic response to infection. Depending on the circumstances, different tissues are activated in a joint effort to fight infection. Otherwise, the JAK/STAT pathway plays an important role during development, and strong *upd1* mutant alleles are lethal during embryonic stage.[28] However, *upd2* and *upd3* single or double mutants are viable and do not show any obvious developmental defects[25] but they encode cytokines that are essential for inter-tissue communication during infection and wounding.

In an attempt to further test the conclusion that hemocytes are the main contributors of Upd2 and Upd3 production upon wasp infection, we tried to ablate circulating hemocytes by expressing an apoptosis gene, *grim*, with the hemocyte-specific *Hml*-GAL4 driver. Surprisingly, we found that the apoptotic hemocytes induced JAK/STAT activation in muscles (Fig. 2A-B). However, this is in line with the observation that apoptosis in hemocytes also induces lamellocyte formation, a hallmark of an activated cellular immune response.[29] Similarly, when we artificially activated the JNK or p38 signaling pathways, or even JAK/STAT itself, in hemocytes, we saw a JAK/STAT response in the muscles.[12] As a result, hemocytes are activated and lamellocytes are generated.[11] This may appear paradoxical, since the cellular immune response is essentially normal even when the JNK, p38 or JAK/STAT signaling pathways are blocked in these cells.[12] Apparently, cytokine production can be triggered by several different perturbations of the signaling systems in hemocytes. The cytokines, Upd2 and Upd3, will then transmit a danger signal to other tissues, where the effect on the somatic musculature is particularly important for the cellular immune response.

Local cross-talk between intestine and visceral muscles

Although it is still not clear how somatic muscles of the *Drosophila* larva affect the cellular immune response, it is better understood how muscles control local responses in the *Drosophila* intestine, where visceral muscles are tightly connected with intestinal trachea, neurons and

the gut itself. These tissues cross-talk in an intimate local interaction during gut regeneration and immune response. Visceral muscles secrete several proteins that non-autonomously regulate regeneration in neighboring tissues. For instance, Wingless (Wg), a ligand for the Wnt pathway, is secreted by the visceral muscles, regulating intestinal stem cell maintenance and proliferation under damage or stress.[30,31] Furthermore, during enteric infection or damage, gut epithelial cells (enterocytes) and their precursors (the enteroblasts) secrete Upd3, which activates JAK/STAT signaling in the visceral muscles and in the enteroblasts themselves. This triggers the visceral muscles to secrete Vein (Vn), one of the EGFR pathway ligands. The stimulated enteroblasts secrete a second EGFR ligand, Spitz (Spi). Together, Vein and Spitz remotely activate EGFR signaling in the intestinal stem cells, stimulating stem cell proliferation.[26,32–35]

To test if similar mechanisms are involved when skeletal muscles activate hemocytes, we assayed the expression of the genes for 4 known *Drosophila* EGFR ligands, Vein, Spitz, Keren and Gurken, in the skeletal muscles, but none of them was significantly induced in wasp-infected larvae (Fig. 2D). To test the role of Wnt signaling we also assayed *Wnt4*, the only Wnt-like gene identified as a JAK/STAT target in *Drosophila* tissue culture cells,[36] with a negative result. Nor could we detect a significant effect on the *Ilp6* gene, encoding an insulin-like peptide (Fig. 2D). Thus, the question remains how JAK/STAT signaling in the skeletal muscles promote the cellular immune response during wasp infection.

Systemic effects of somatic muscles on general physiology

Several studies during the past decade have demonstrated a major role of skeletal muscles in systemic responses to exercise, stress and aging, in mammals as well as in the model organisms *Drosophila melanogaster* and *Caenorhabditis elegans*.[37-39] Muscles secrete myokines and metabolites with major systemic effects on metabolism, controlling physiological homeostasis, aging and disease progression. The role of these factors in immunity is less well understood.

In *Drosophila*, suppression of the insulin pathway in skeletal muscles leads to reduced muscle size and decreased feeding behavior, which finally affects the growth of other tissues.[40] Overexpression of FOXO and its target 4E-BP in skeletal muscles not only prevents aging in muscles but also in other tissues, by decreased accumulation of protein aggregates and increased autophagy.[41,42] Recently Demontis *et al.*[43] also reported that Myoglianin, a myokine, plays an important role in tissue communication between *Drosophila* skeletal muscles and adipose tissue during aging.

In mammals, interleukin-6 (IL-6) is perhaps the most thoroughly studied myokine.[37] IL-6 was found to be secreted in vast amounts by human skeletal contracting muscles during exercise, causing plasma levels to increase by almost 2 orders of magnitude.[44,45] Not only exercise but also other stimuli, such as bacterial lipopolysaccharides, reactive oxygen species, and inflammatory cytokines, induce IL-6 production from skeletal muscles.[46-49] Secreted IL-6 exerts a positive or negative effect on metabolism and insulin sensitivity, depending on the physiological or disease context.[39] Other myokines, including insulin-like growh factor 1(IGF-1), IL-8, IL15, and meteorin-like (Metrnl), with different effects on physiology, were shown to be secreted by muscles.[37,50] Interestingly, Metrnl was reported to affect the immune system. Metrnl, induced by exercise training, does not only regulate energy homeostasis, but this factor also stimulates several immune cell subtypes to enter the adipose tissues and secrete anti-inflammatory factors.[50]

By analogy to these observations it is possible that the somatic muscles in *Drosophila* may control immunity by secreting still unknown myokines that act directly on the immune cells. Alternatively, they may affect immunity indirectly, by acting as a master switch of metabolism. The skeletal musculature would then be a central player in a systemic response, regulating nutrient availability according to the needs of the cellular immune defense. A similar mechanism may also be involved in the wasting of muscles and adipose tissue seen in human chronic disease.

Toll signaling and cellular immunity

The fat body is a bulky and dominating tissue in the growing larva, with important functions for fat and protein storage and for general metabolism.[51] It is also important in the humoral immune response, being the main site of synthesis of antimicrobial peptides. The role of the Toll pathway in this response is well documented.[1,2,4,52–54] More recently it has become clear that the fat body can also activate a hemocyte response in *Drosophila* larvae, and that this effect also depends on

activation of Toll signaling in the fat body.[13] It had previously been shown that constitutively active *Toll* gain-of-function mutants such as $Toll^{10b}$ show signs of an activated cellular immune response, for instance, loss of sessile hemocytes, lamellocyte formation and formation of melanotic masses of activated hemocytes, while loss-of function *Toll* mutants have a reduced capacity to encapsulate parasitoid wasp eggs.[5,8–10] It is sufficient to express the $Toll^{10b}$ mutant in hemocytes to generate a *Toll* gain-of-function phenotype,[11] but recently it was found that artificial Toll activation in other tissues, notably in the fat body, gave an even stronger hemocyte activation phenotype.[13] The hemocyte phenotype of the $Toll^{10b}$ mutant, expressed under its endogenous *Toll* promoter, was suppressed when downstream components in the Toll pathway were knocked down in fat body, but not in hemocytes.[13] These results suggest that Toll activation in the fat body is a more important factor for the cellular immune response than Toll activity in the hemocytes themselves.

The involvement of muscles and fat body in the cellular immune response is schematically depicted in Figure 3. While JAK/STAT activity in the somatic muscles is necessary and probably sufficient for a strong cellular immune response, Toll signaling in the fat body is sufficient to trigger hemocyte activation, but it is not absolutely necessary for the defense against wasp eggs. Only a minority of wasp-infected larvae shows a strong Toll response, but a positive correlation was seen between the Toll response and the ability to fight the infection.[13] Interestingly, a more consistent Toll response was seen in larvae that the wasp had stung, without injecting an egg. This suggests that together with the egg, the wasp injects an inhibitor of Toll activation.

The ligand of the receptor Toll is the TGFβ-related cytokine Spätzle, which is constitutively secreted by hemocytes. It can be further induced, for instance by bacterial infection,[55] but it must be processed by proteolytic cleavage before it is active.[56] In the *Drosophila* embryo, Spätzle is processed by the serine protease Easter while a related protein, the Spätzle-Processing Enzyme (SPE), does the same job in the hemolymph. Both are in turn activated by complement-like serine protease cascades,[4,54] but it is not clear how, or if, Spätzle is processed in the context of a wasp infection. Nor is it clear how Toll signaling in the fat body leads to a mobilization of the cellular immune response. One interesting candidate signaling molecule is Edin, which is secreted by the fat body in response to wasp infection and required for mobilization of sessile hemocytes, but not for lamellocyte formation.[57,58]

Other tissues involved in the immune response

Further examples of tissue interactions in the cellular immune response of *Drosophila* have been reported recently, for instance between neurons and sessile hemocytes and between fat body and brain.[57] During larval development, specific neurons in the peripheral nervous system supply a microenvironment in "pockets" between the epidermis and the somatic muscles. These subepidermal sites attract colonizing hemocytes

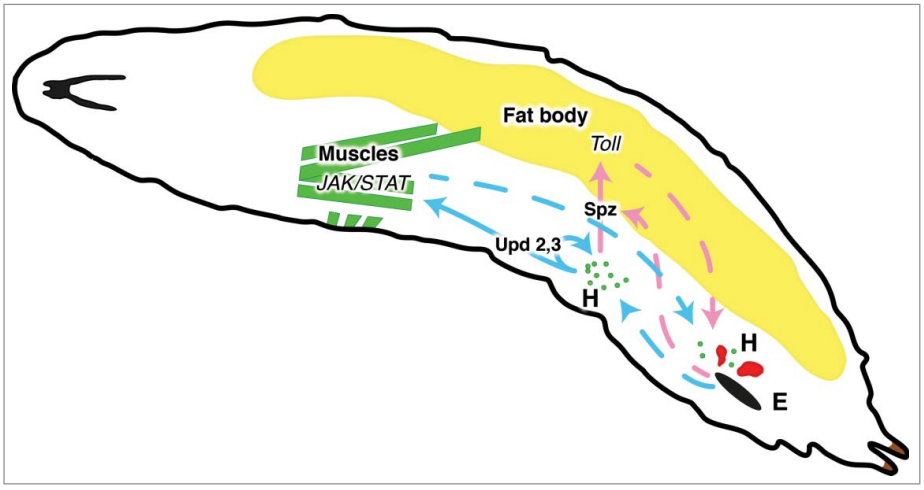

Figure 3. Schematic representation of interactions between a wasp egg (E), hemocytes (H), muscles and fat body. Plasmatocytes are shown in green, lamellocytes in red. Interactions via identified cytokines are shown as solid arrows. Other interactions (which may be indirect) are shown as dashed arrows.

and provide inductive factors for hemocyte expansion. These resident hemocytes co-localize with particular peripheral neurons and the ablation of these neurons leads to loss of resident hemocytes.[59] Apparently, communication between peripheral neurons and sub-epidermal hemocytes is critical for the maintenance of this sessile hemocyte population.

Furthermore, high dietary fat and sugar can induce Upd2 secretion from the fat body.[60] Secreted Upd2 indirectly stimulates insulin-producing cells in the brain to release insulin, which systemically affects growth and metabolism.[60] It has also been reported that a lipid-rich diet stimulates *Drosophila* hemocytes to secrete Upd3, which systemically impairs glucose homeostasis and reduces lifespan.[61]

Conclusions

It is clear that the response to an infection in *Drosophila* larvae, for instance by a parasitic wasp, is not a local phenomenon that is restricted to a limited number of specialized cells of the immune system. On the contrary, it is a major event that affects the physiology of the entire organism and it involves all major organ systems in a concerted systemic immune response. The findings we have discussed here may also have relevance for the relationship between physical activity, nutrition, life span and immunity, in flies as well as in man.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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