論文の内容の要旨

論文題目

Genetic control of autoimmune activation in *Drosophila*

apoptosis-deficient mutants

(ショウジョウバエ細胞死変異体に見られる自己免疫反応の遺伝学的解析)

明　銘

[Background and introduction]

Inflammatory response represents a critical aspect of *in vivo* defense system, which can be triggered in response to threat with both intrinsic and extrinsic origin. Upon microbial challenge, an inflammatory response is essential in eliminating invading pathogens and wound repair. However, unresolved, chronic inflammation could be detrimental to the host, therefore should be tightly regulated to avoid further damage to the tissues involved. Recently, accumulating evidence has suggested that inflammation could also be induced by a handful of host factors in the absence of microbial signals, causing pathological symptoms as seen in stroke, atherosclerosis, diabetes and so on. It is of great importance to understand the nature of the endogenous immunogen and the molecular basis for such sterile inflammatory response.

In this study, *Drosophila*, with its vast collection of genetic tools and analyzing techniques available, is utilized to decipher this phenomenon *in vivo*. The relatively simple composition of body tissues makes them well fitted for studies at organismal level, whereas it is difficult to investigate the inter-tissue communication *in vivo* in mammalian system. More importantly, although devoid of the highly specific adaptive immunity that governs a great part of the mammalian immune system, *Drosophila* possesses an effective and conserved innate immune system that actually holds the key to multiple aspects in defense response, thus has become an attractive tool in elucidating the fundamental principles in immunity.

Apoptosis, a well-conserved mechanism of cell suicide, is considered immuno-silent and plays fundamental roles in physiological processes such as morphogenesis, homeostasis as well as host defense. In *Drosophila*, Dronc, the only initiator caspase, is the key regulator for most apoptosis; mutants for *dronc* exhibit severe defects and die at mid-pupal stage. In the present study, I conducted a detailed characterization of sterile inflammation on the basis of genetic manipulation in *Drosophila dronc* null mutants and demonstrated that canonical Toll signaling is responsible to mount a potent response to endogenous immunogenic factors released upon apoptosis deficiency, and postulate that *Drosophila* caspase mutants would serve as a model to enable further elucidation of the mechanism of sterile inflammation *in vivo*.

[Methods and Results]

1. Sterile inflammation manifested as systemic activation of Toll pathway in *dronc*$^{\Delta A8}$ mutants

Previously a deletion line for *dronc* was generated by P element mobilization in my laboratory. The homozygous mutants are refractory to apoptotic stimuli and show complete lethality during pupal development. With this *dronc*$^{\Delta A8}$ mutant line, I then sought to explore whether inflammatory response ensues due to the apoptosis deficiency.

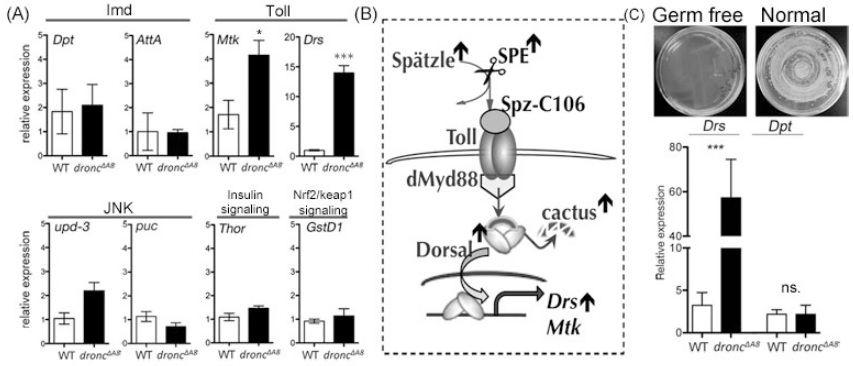

Figure 1. *dronc*$^{\Delta A8}$ mutants display hyper-activated Toll signaling in the absence of bacterial infection. (A) Relative induction of stress responsive genes was analyzed by quantitative RT-PCR. (B) Toll signaling in *Drosophila*. Arrows indicate the enriched signaling proteins in mutants. (C) Germ free animals was verified by LB plating and the activation of immune pathways was assessed using qRT-PCR. * $p<0.05$, *** $p<0.001$

In third instar larvae, quantitative RT-PCR results revealed a profound induction of the Toll signaling, one of the prominent immune regulators induced by fungi and gram-positive bacteria, with significantly elevated production of Drosomycin (*Drs*, an anti-fungal peptide frequently used as a hallmark of Toll pathway activation in *Drosophila*), and to a lesser extent, *Metchnikowin (Mtk*, an anti-microbial peptide) (Figure 1A). On the contrary, Imd pathway, the other *Drosophila* immune signaling activated mainly by gram-negative bacteria, remains unaffected. Likewise, no discernable induction could be observed for the other stress pathways tested. Moreover, in *dronc*$^{\Delta A8}$ mutants, the concomitant elevation of the signaling molecules in Toll pathway demonstrates a robust and systemic activation of this pathway (Figure 1B). Given the important role of Toll signaling in combating invasive pathogen, I attempted to determine whether microbial infection plays a part by looking at the immune activation in germ free animals (Figure 1C). The qRT−PCR results showed that removing the bacteria load didn't abolish Toll activity, indicating in *dronc*$^{\Delta A8}$ mutants, it is the host factors, rather than bacterial elicitors, that trigger the immune activation.

2. Apoptotic deficiency causes constitutive NF-κB activation.

In metazoan, caspases are produced as inactive precursors and become activated by upstream caspase through proteolytic cleavage, which depends on an active cysteine site. I then assessed whether the catalytic activity of Dronc is crucial in maintaining immune-homeostasis.

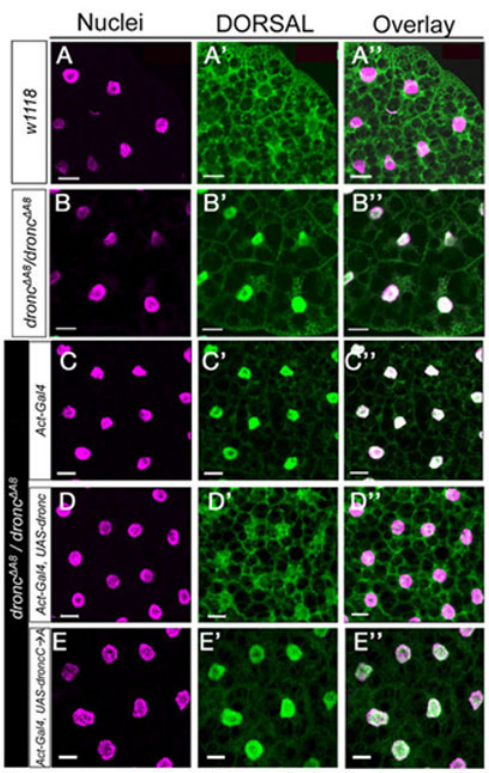

Figure 2 Immuno-staining of fat body reveals intracellular localization of the Rel protein Dorsal for Toll pathway. Genetic background: a: w1118, b: *dronc*$^{\Delta A8}$, c: Act-Gal4/+; *dronc*$^{\Delta A8}$, d: Act>*dronc*^{WT}; *dronc*$^{\Delta A8}$, e: Act>*dronc*$^{C->A}$; *dronc*$^{\Delta A8}$. Scale bar: 20μm

Drosophila fat body, the functional equivalent of mammalian liver, is the central tissue for systemic production of AMPs, which are transcriptionally regulated by corresponding NF-κB/Rel factors. Accordingly, in mutant fat body, I could detect substantial amount of nuclear localized Dorsal, the Rel protein downstream of Toll pathway, suggesting a constitutive activation of Toll intracellular cascade (Figure 2A-B). Furthermore, Dorsal immuno-staining demonstrates that oversxpression of wild type *dronc* could readily reverse Dorsal nuclear import in mutant background (compare figure 2C-D), whereas a loss of function allele (*dronc C→A,* Figure 2E*)* with an alanine substitution at the active cysteine site couldn't. These results suggest that catalytic activity is crucial to suppress the Toll signaling activation.

3. In *dronc*$^{\Delta A8}$ mutant, Toll pathway activation is mediated by active form of the cytokine-like factor Spätzle.

In infectious activation of Toll, extracellular recognition of stimuli leads to the processing of the endogenous cytokine Spätzle to its active form that comprises C-terminal 106 amino acids, which could bind Toll receptor with high affinity and relay the signal intracellularly via recruiting the adaptor protein dMyd88 (Figure 1B). I then explored whether, in *dronc*$^{\Delta A8}$ mutants, host factor-directed NF-κB activation is mediated through such canonical cascade. By genetically combining the mutation for either *spz* or *dMyd88* with *dronc*$^{\Delta A8}$ background, I showed that both factors are essential in mediating Toll signaling, for the induction of *Drs* was completely abolished in individuals doubly mutated for *dronc* and immune factors (Figure 3A).

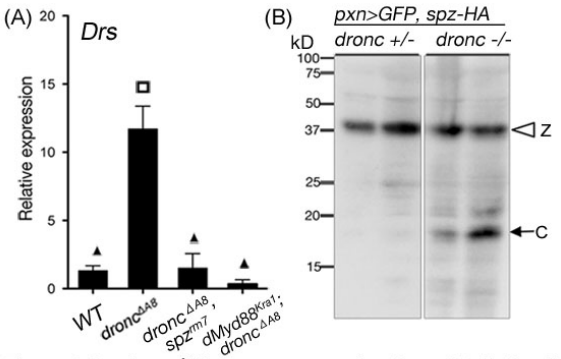

Figure 3 In *dronc*$^{\Delta A8}$ mutants, transduction of Toll signal requires both the cytokine-like ligand Spätzle and the cytosolic adaptor dMyd88. (A) Mutation in either *spz* or *dMyd88* abolished *Drs* induction under *dronc*$^{\Delta A8}$ genetic background. Between different mark: p<0.01, between identical mark: p>0.05. (B) Western blotting detects active form of the Spz in *dronc*$^{\Delta A8}$ hemolymph. Z: full-length zymogen, C: cleaved form.

As genetics indicates *spz* is necessary to mediate Toll signaling, I next tried to determine whether the proteolytic processing occurs in the mutant hemolymph. To this end, I overexpressed tagged Spätzle protein (Spz-HA) using a hemocytes driver (pxn-Gal4) and assessed its cleavage in hemolymph by western blotting (Figure 3B). The active Spz was detected as a 19kDa protein in mutant hemolymph. Taken together, these results suggest that impairment in caspase leads to the processing of the *Drosophila* cytokine Spätzle, causing constitutive activation of the canonical Toll signaling.

4. Inactivation of Toll signaling rescues hemocyte defects.

In addition to humoral immunity that is characterized by AMP secretion from the fat body, the cellular immunity represents another vital force in *Drosophila* host defense system. In third instar larvae, two distinct fractions of hemocytes could be observed: circulating hemocytes that reside in the hemolymph, and the sessile hemocytes that attach to the inner surface of the integument. In an effort to reveal whether loss of *dronc* could influence cellular immunity, I observed a significant elevation in number of circulating blood cells in mutant larvae, which could be partially attributed to Toll activation (Figure 4A). In addition, the mutants exhibit a profound disruption of the banded pattern for sessile hemocytes, as visualized by GFP-expressing blood cells through their translucent skin (Figure 4B). Taken together, the above results implicate caspase function in the regulation of hematopoietic tissue, likely through Toll signaling.

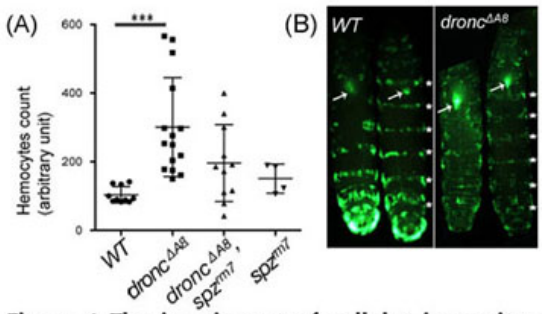

Figure 4 The impairment of cellular immunity. (A) Circulating hemocyte counts from indicated genotypes indicates a drastic hyperplasia in *dronc*$^{\Delta A8}$ mutant. (B) Disruption of the banded pattern for sessile hemocytes. blood cells associated with the integument are visualized using pxn-Galr4, UAS-GFP. Band organization is indicated by '*'; arrows point to the hematopoietic organ-lymph gland.

[Summary and discussion]

In addition to microbial determinants, there is increasing evidence suggesting that host factors would activate immune system as a result of environmental or physiological stressor. In this study, I characterized a sterile inflammatory response with respect to both humoral and cellular immunity using *Drosophila* mutants for the initiator caspase, Dronc. The results suggest that caspase activity might be required to maintain homeostasis in terms of suppressing chronic immune signaling. In *dronc*$^{\Delta A8}$ larvae, Toll innate immune signaling is specifically induced following the generation of the active form of the cytokine Spätzle. This phenomenon shows profound analogy to the induction of TLR signaling by altered or abnormal host factors in mammalian system. The fundamental role of TLR/IL-1 signaling in endogenous factor-induced host response is starting to be understood, and *Drosophila dronc*$^{\Delta A8}$ mutants provide us with a genetic accessible tool that enables elucidation of the molecular basis of sterile inflammation *in vivo*.

In vertebrate system, in addition to an induced production of pro-inflammatory cytokines and chemokines from the effected site, sterile inflammation is also marked by the extensive activation and recruitment of immune cells such as neutrophils and macrophages. *Drosophila* hemocytes constitute the major body of mobile immune cells, and are indispensible for the cellular arm of defense response. In a manner similar to mammalian immuno-surveillance cells, *Drosophila* hemocytes play significant parts during host defense response and maintenance of homeostasis under normal state. In $dronc^{\Delta A8}$ mutant, the profound changes of behavior associated with these insect blood cells exemplified a simultaneous disturbance of the cellular immunity by sterile factors originated from the host.

The physiological interaction between apoptotic cell death and inflammatory response, two of the most important biological processes in organisms, underlies pathological principles of human diseases such as cancer growth and massive degeneration, thus has been under intensive investigations. In vertebrate models, multiple stimuli are shown to converge on caspase function to promote inflammatory responses. Overall, the results of present study representing a non cell-autonomous role of apoptotic caspase in suppressing inflammation might highlight a novel connection between cell death control and inflammation.