Biochemical and Biophysical Research Communications 430 (2013) 1120–1125

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Activation of Imd pathway in hemocyte confers infection resistance through humoral response in *Drosophila*

Bryce Nelson [a,1], Tina Freisinger [a,b], Kenichi Ishii [c], Kiyoshi Okado [a,d], Naoaki Shinzawa [a,c,2], Shinya Fukumoto [a], Hirotaka Kanuka [a,c,d,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan
[b] Max Planck Institute of Biochemistry, Martinsried, Germany
[c] Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan
[d] Department of Tropical Medicine, The Jikei University School of Medicine, Tokyo, Japan

ARTICLE INFO

Article history:
Received 1 December 2012
Available online 19 December 2012

Keywords:
Infection
Bacteria
Innate immunity
Drosophila
Antimicrobial peptide

ABSTRACT

Upon microbial invasion the innate immune system of *Drosophila melanogaster* mounts a response that comes in two distinct but complimentary forms, humoral and cellular. A screen to find genes capable of conferring resistance to the Gram-positive *Staphylococcus aureus* upon ectopic expression in immune response tissues uncovered *imd* gene. This resistance was not dependent on cellular defenses but rather likely a result of upregulation of the humoral response through increased expression of antimicrobial peptides, including a Toll pathway reporter gene *drosomycin*. Taken together it appears that Imd pathway is capable of playing a role in resistance to the Gram-positive *S. aureus*, counter to notions of traditional roles of the Imd pathway thought largely to responsible for resistance to Gram-negative bacteria.

1. Introduction

Study of the innate immune system of *Drosophila* has garnered much information concerning underlying mechanisms of resistance to microbial invasion, not only in insects but due to high conservation, also in humans. Upon entry into a fly, a microbe is faced with cellular defenses comprised largely of phagocytosis by the fly equivalent of macrophages, plasmatocytes, followed by autophagy [1–3]. Any remaining microbes not phagocytosed are faced with a second wave of defense in the form of humoral defenses that include melanization and a spectrum of antimicrobial peptides (AMPs) secreted from various tissues, including hemocytes and the fat body, an analog to the mammalian liver [4]. These two major forms of resistance are connected; a full humoral response requires not only phagocytosis of the microbe but also digestion within the phagocytic hemocyte [5].

Controlling these responses are two highly conserved pathways that lead to activation of NF-κB-like transcription factors and subsequent transcription of immune related genes. The Toll pathway responds to microbial invasion through activation of the Toll-like receptor, Toll, by a processed ligand, Spatzle, that in turn activates a cassette of proteins consisting of Tube, Pelle, and dMyD88 [6–10]. Once activated, this complex is able to trigger the degradation of the I-κB protein, Cactus, that sequesters the NF-κB transcription factors Dif and Dorsal in the cytoplasm [11–13]. Another pathway, the Imd pathway, mounts an immune response through its transcription factor Relish whose nuclear translocation is triggered by Imd, a homolog of the tumor necrosis factor receptor-interacting protein, RIP, through downstream components including IKKa, IKKβ, dTAK1, DIAP2, dFADD and the Caspase-8 homolog, Dredd [14–22].

Initial work delineating these two cascades posited that the Toll pathway was responsible for mounting defenses against Gram-positive and fungal invasion while the Imd pathway was required for full response to Gram-negative bacteria. While much of this work has provided a foundation that clearly points to pathway discrimination recent work has revealed a more complicated picture that appears to involve each specific microbe able to elicit unique but overlapping responses [23,24]. For instance, Imd pathway mutants have shown increased sensitivity to the fungus *Beauveria bassiana* as well as to the Gram-positive bacteria *Streptococcus pneumonia* [25,26]. Furthermore, although disruption of Toll signaling has no discernible effect on survival to the Gram-negative *Escherichia coli*, double mutants combining mutations to both pathways show increased sensitivity compared to Imd pathway mutants [27,28]. Reciprocally, increased sensitivity to the Gram-positive bacteria

* Corresponding author. Address: Department of Tropical Medicine, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan. Fax: +81 3 3431 4459.
E-mail address: kanuka@jikei.ac.jp (H. Kanuka).
[1] Present address: Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Ontario, Canada.
[2] Present address: Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.027

Table 1
Candidate genes conferring resistance to *S. aureus* upon expression in immune tissues.

GS line	Gene	Molecular information
GS9049	CG5576 (*imd*)	Immune response
GS7366	CG14434	Adenylate cyclase activity
GS7386	CG9397 (*jing*)	Transcription factor activity
GS7392	CG14618	Unknown function
GS8163	CG32717 (*stardust*)	Guanylate kinase activity
GS5130	CG9613 (*Coq2*)	4-Hydroxybenzoate octaprenyltransferase activity
GS5176	CG14789 (*O-fut2*)	Peptide-O-fucosyltransferase activity
GS8162	CG11940 (*jog*)	Signal transduction
GS7480	CG1689 (*Lozenge*)	Transcription factor activity
GS9860	CG6556 (*cnk*)	Ras protein signal transduction

Enterococcus faecalis was seen by double pathway mutants compared to Toll pathway mutants [27].

These findings suggest a complicated picture wherein both pathways work synergistically to contribute to the survival of the infected fly [23,29]. The picture becomes increasingly complicated upon examination of AMP expression where microbial cell wall peptidoglycans and glucans appear to act as a primary source of pathway activation [27,30]. DAP-type peptidoglycans found mainly in Gram-negative bacteria or lysine-type peptidoglycans found primarily in Gram-positive bacteria are linked to pathway activation through peptidoglycan recognition proteins (PGRP-LE and PGRP-SA for the Imd and Toll pathways, respectively). Furthermore, glucan-binding Gram-negative binding proteins, such as GNBP1, which acts in combination with PGRP-SA for Toll pathway activation, work in concert with the peptidoglycan recognition proteins to achieve full AMP expression [31–33]. Complicating the picture even further are recent findings suggesting another Toll pathway peptidoglycan recognition protein (PGRP-SD) is able to bind DAP-type peptidoglycan [34]. Taken together a complicated picture emerges wherein the Toll and Imd pathways respond according to the specific invading microbe rather than in terms of Gram staining.

A gain-of-function screen revealed a role for *imd* gene in resistance to *Staphylococcus aureus*, again running counter to traditional notions of Imd pathway resistance. This resistance was not dependent on cellular responses but rather likely due to the broad spectrum of AMP activity suggesting Imd pathway control over this humoral response.

2. Materials and methods

2.1. Fly lines

Flies were raised on standard *Drosophila* medium at 25 °C. w^{1118} flies were used as wild type lines. *UAS-lacZ* lines were used as a second control in all experiments with similar results. All GS strains were provided from Kyoto *Drosophila* Genetic Research Center. *UAS-imd*^{2} (a gift from Dr. Jean–Marc Reichhart) was used for all experiments except for *UAS-imd*^{S} which was isolated from the GS library screen (*GS9049*). Both lines resulted in resistance to *S. aureus*. *peroxidasin-GAL4* line was a gift from Dr. Micheal J. Galko. *diptericin-lacZ drosomycin-GFP* was a gift from Dr. Bruno Lemaitre. *drosomycin-GFP* was a gift from Dr. Jules Hofmann. *drosocin-GFP*, *attacin-GFP*, and *diptericin-GFP* were gifts from Dr. Jean–Luc Imler. *cecropin-lacZ* was a gift from Dr. Ylva Engstrom. *UAS-dsRNA* lines were obtained from NIG-Fly Stock Center, Japan [*tube* (10520R-1), *imd* (5576R-1), *POSH* (4909R-1), *dTAK1* (1388R-1), *key* (16910R-4), *dFADD* (12297R-1)] and the Vienna *Drosophila* RNAi Center [*dMyd88* (25399), *dorsal* (45996), *dif* (30579), *Caspar* (35419), *relish* (49413), *dredd* (28041), *TAB* (37553), *IKK*β (26427), *PGRP-LC* (51968)].

2.2. Gain-of-function screening

To identify genes enhancing infection resistance for *S. aureus* pathogenicity we adapted the *P*-element-based gene search (GS) system [35]. The GS vector contains the UAS enhancer adjacent to a core promoter. In this screen, genes are detected on the basis of phenotypic changes caused by the GAL4-dependent forced expression of the vector-flanking DNA. This system has greater efficiency than others that are presently used for gain-of-function screens [35]. For the first screen, females of the *pxn-GAL4* genotype were crossed to males from each line in the GS collection. F1 progeny were injected with *S. aureus* (described below) and survival rates were monitored daily. Genomic DNA regions flanking P element of the GS vector were recovered from these GS lines by standard inverse PCR protocols (http://www.fruitfly.org/about/methods/inverse.pcr.html). The recovered genomic fragments were sequenced and analyzed with BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) databases to identify the candidate genes (Table 1).

2.3. Bacterial strains

S. aureus was provided from Dr. Hiroshi Hamamoto and Dr. Kazuhisa Sekimizu [36] and was cultured at 37 °C in the dark without agitation in LB broth medium. *Salmonella typhimurium* (SL1344) was a gift from Dr. David Schneider and cultured at 37 °C in the dark without agitation in LB broth medium containing 100 μg/ml streptmycin.

2.4. Infection assays

Flies were anesthetized with CO_2 and injected with the indicated strain of bacteria in medium. Injection was carried out using an individually calibrated pulled glass needle attached to IM-300 microinjector (Narishige). Flies were always injected in the abdomen, close to the junction with thorax and just ventral to the junction between the ventral and dorsal cuticles. After injection, flies were transferred to fresh vials once every three days. Sixty-nine nanoliters of overnight *S. aureus* cultures diluted to an OD_{600} = 0.005 in LB media were injected into the abdomens of 15 newly eclosed male flies and incubated at 25 °C. At least three independent assays were performed for each experiment.

2.5. β-Galactosidase assay

Five adult flies (day 1) were crushed in 250 μl LacZ buffer (100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) with a plastic pestle and 100 μl of solution was transferred in replicate into tubes each containing 500 μl ONPG buffer (LacZ buffer plus 2-mercaptoethanol and *o*-nitrophenyl-β-D-galactopyranoside (ONPG)). Timing started upon transfer and stopped with addition of 100 μl 1 M Na_2CO_3 to stop the reaction. Reactions were carried

out at room temperature. OD was measured at 415 nm and units were displayed as arbitrary units using the formula: 1000× OD_{415}/time (min).

2.6. Phagocytosis blocking assay

Flies were injected with latex beads (0.2 μm red fluorescent latex beads, F-8763, Invitrogen) (or sterile PBS for mock treatment) in order to block phagocytosis as described previously [37] 30–60 min prior to injection with *S. aureus* (for infection assays) or 69 nl of fluorescently labeled heat–killed *S. aureus* (S-2851, Invitrogen) to monitor phagocytic block. Flies injected with fluorescently labeled bacteria were incubated 30 min before injection with 138 nl Trypan blue to quench the signal of non-phagocytosed bacteria before visualization and quantification of fluorescence [37].

2.7. Bacterial clearance assay

Adult flies (day 1) were injected with *S. aureus* as per infection assay and incubated for the indicated times. Ten flies per sample were ground into 100 μl of 10 mM $MgSO_4$ before addition of 900 μl of LB and serial diluted before plating onto LB media. Each time point was the average of at least four replicates.

2.8. Phenoloxidase activity assay

Assay was performed as described previously [38].

2.9. AMP reporter assay

A line containing *drosomycin-GFP*, *diptericin-lacZ*, *peroxidasin-GAL4*, and *UAS-imd* was crossed to the various *UAS-dsRNA* lines or wild-type (both w^{1118} and *SwedishC*). Flies were grouped together as strong signal, weak signal, or no signal. Crosses to wild-type lines were normalized to 100% signal (combination of strong and weak signal) and all other lines, crossed in parallel, were quantified relative to the wild-type lines. At least 300 flies were counted from each sample.

2.10. Northern blotting

Total RNA was isolated from twenty flies (day 1) using TRIzol reagent (Invitrogen). mRNA expression of antimicrobial peptide (AMP) genes (*diptericin* and *drosomycin*) and *rp49* was analyzed by RNA blotting as described previously [39]. Samples were performed in duplicate with similar results.

2.11. Statistical analysis

Survival curves were subjected to a Mantel–Cox log-rank analysis to determine statistical significance. Nonparametric two-tailed *t*-tests were performed for clearance assays, phenoloxidase activity assays, and *lacZ* reporter activity assays. All results are indicated in the appropriate figure legends except phenoloxidase activity which showed no statistical significance.

3. Results

To dissect host factors mediating humoral and cellular defenses in immune response tissues, a gain-of-function fly library (Gene Search (GS) system) was infected with the extracellular pathogenic Gram-positive bacterium, *S. aureus*, and survival rates were measured. We screened *Drosophila* GS lines in combination with the hemocyte-expressing GAL4-expression fly strain (*peroxidasin* (*pxn*)-*GAL4*), which results in the ectopic hemocyte expression of one or two genes from the *Drosophila* genome [35]. Despite initial reports of *pxn-GAL4* expression being hemocyte specific, weak expression was seen in larval fat body cells as was shown recently (data not shown and [40]). Surprisingly, expression of *imd*, an Imd pathway component and homolog to mammalian RIP, led to significant resistance of adult flies (Fig. 1A and Table 1). Overexpression of the death domain-containing Imd has been shown to lead to cell

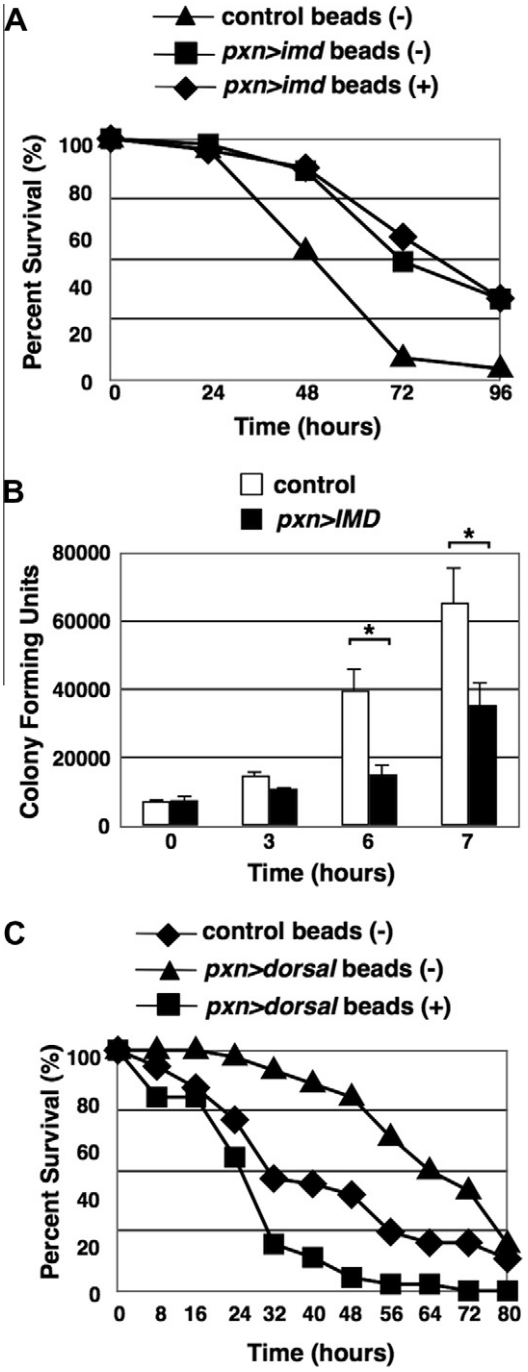

Fig. 1. Overexpression of *imd* in hemocytes leads to *S. aureus* resistance. (A) One day old wild-type or *imd*-expressing flies (*pxn* > *imd*) were injected with PBS before injection with *S. aureus* ($p < 0.0001$ for wt versus *imd*-expressing flies). Indicated flies were injected with latex beads to block phagocytosis prior to infection ($p < 0.0001$ for wt versus phagocytotic blocked *imd*-expressing flies). (B) CFUs counts of wild-type and *imd*-expressing flies after injection with *S. aureus* ($^*p < 0.05$). (C) One day old *dorsal*-expressing flies (*pxn* > *dorsal*) were injected with latex beads to block phagocytosis prior to injection with *S. aureus* as shown in (A).

death [19,41], however, *imd*-expressing hemocytes appeared to be healthy (data not shown).

Increased resistance to microbial infection can be achieved through two major mechanisms: tolerance whereby the microbe is sequestered and the stress of infection is coped or via resistance involving bactericidal (killing of bacteria) or bacteriostatic (proliferation inhibition) mechanisms [42,43]. In order to distinguish between these two mechanisms of survival, colony forming units (CFUs) were quantified from both wild type and *imd*-expressing flies after infection with *S. aureus*. A steady increase in CFUs could be seen in both lines, however, *imd*-expressing lines showed consistently lower numbers of CFUs compared to wild type lines suggesting prolonged survival was likely derived from resistance mechanisms (Fig. 1B).

The innate immune response of organisms can be divided generally into humoral and cellular responses; phagocytosis is a major component of cellular defense to microbial infection and was therefore tested to determine the extent to which cellular defenses were required for the observed resistance. Blockage of phagocytosis prior to infection had no effect on survival rates of *imd*-expressing resistant flies (Fig. 1A), however, phagocytotic blockage made *dorsal*-expressing flies susceptible to *S. aureus* infection (Fig. 1C) suggesting resistance of *imd* expressing flies to *S. aureus* was not dependent on intact cellular defenses.

The humoral response of *imd*-expressing flies seemed the likely mechanism by which resistance was achieved. One form of humoral defense comes through the melanization process that can be quantified through monitoring of phenoloxidase activity. No increase in melanization could be detected upon *imd* expression (data not shown) consistent with previous reports of *imd* independence of the melanization process [31]. Along with melanization, tissues including hemocytes and the fat body secrete AMPs toxic to microbial invaders. Northern blotting was used to assess AMP expression, specifically *diptericin* (*dpt*), a classic Imd pathway AMP, and *drosomycin* (*drs*), controlled by both pathways but used mainly to monitor Toll pathway activity. Dramatic increases in both *dpt* and *drs* expression was detected upon expression of *imd* in immune response tissues (Fig. 2A). Use of *Drosophila* reporters to monitor AMP expression led to similar results with all AMPs tested showing activation upon expression of *imd* in immune response tissues (Fig. 2B and C). Taken together, it appears that expression of *imd* in immune response tissues leads to expression of a broad spectrum of AMPs to levels capable of conferring *S. aureus* resistance.

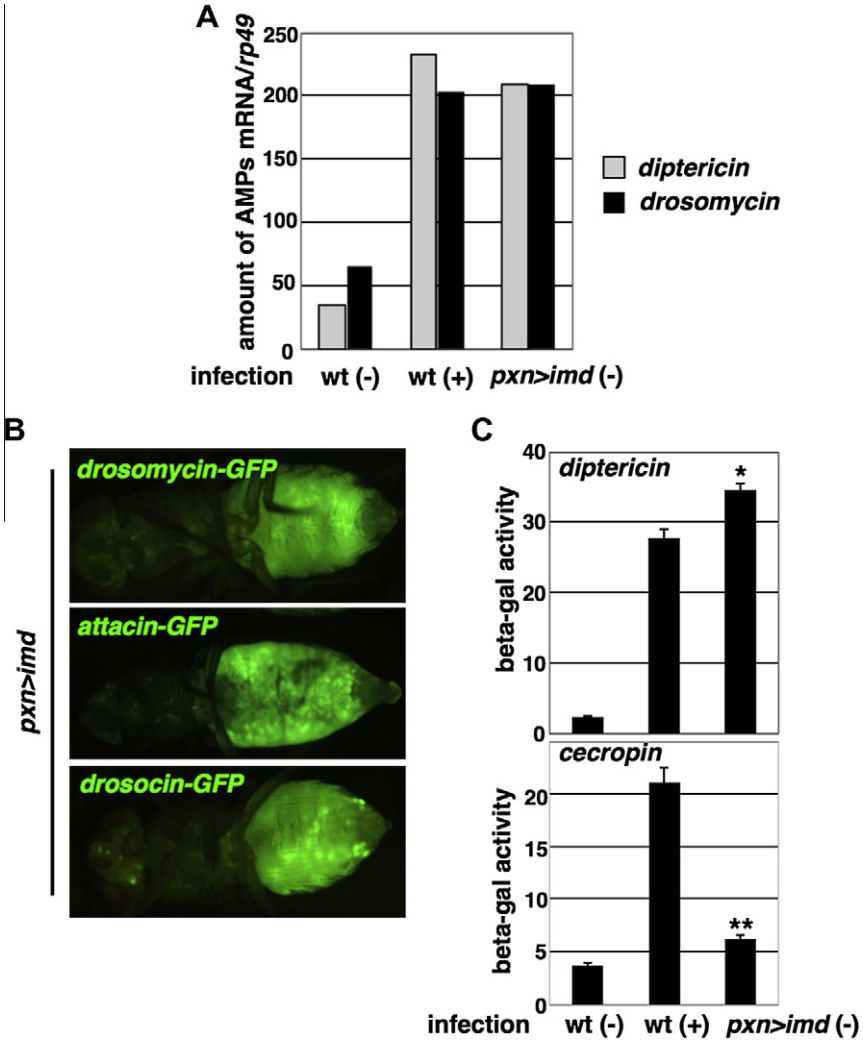

Fig. 2. AMPs activity upon *imd* expression in hemocytes. (A) Northern blot analysis with quantification of *diptericin* and *drosomycin* expression in adult flies. One day old wild-type flies infected with a combination of *S. aureus* and *Salmonella typhimurium* and incubated for 6 h prior to collection were used as a positive control. (B) One day old flies were monitored for *drosomycin-GFP* (upper), *attacin-GFP* (middle), and *drosocin-GFP* (lower) activity. (C) One day old flies were monitored for *diptericin-lacZ* (upper) ($^{*}p < 0.05$ for wt versus *imd*-expressing flies) and *cecropin-lacZ* (lower) activity ($^{**}p < 0.01$ for wt versus *imd*-expressing flies). One day old wild-type flies injected with a combination of *S. aureus* and *Salmonella* 6 h prior to quantification were used as a positive control.

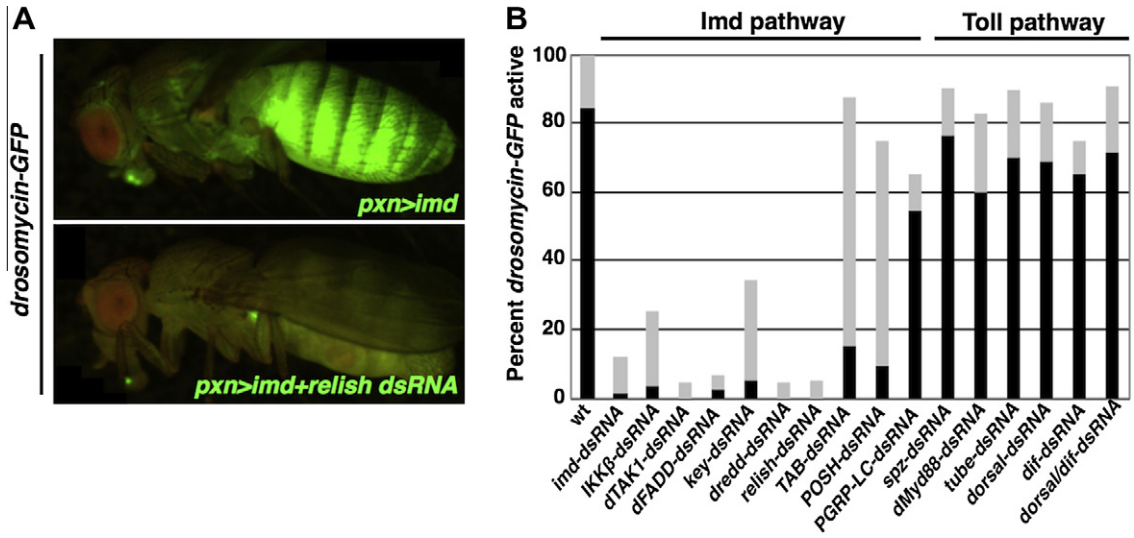

Fig. 3. Imd pathway control of *drosomycin-GFP* expression in *imd* expressing flies. (A) *drosomycin-GFP pxn > imd* flies crossed to *UAS-relish-dsRNA* line. Endogenous *drosomycin-GFP* activity can be seen in the proboscis, abdomen, and genitals. (B) *drosomycin-GFP pxn > imd* flies crossed to indicated *UAS-dsRNA* lines were scored for *drosomycin-GFP* activity (none/moderate in gray, or high in black).

drs has been used traditionally to monitor Toll pathway activity, however, *drs* transcription has been shown to be controlled by both pathways in a tissue dependent manner; the Imd pathway controls *drs* expression in surface epithelial tissues while the Toll pathway is required for its expression in the fat body and salivary glands [44]. Closer examination of *imd* expressing flies and larvae revealed *drs*-GFP activity in both the fat body and salivary glands as well as hemocytes (data not shown). In order to determine which pathway was responsible for the observed *drs* activity components of each pathway were knocked down. Knockdown of Imd components in combination with *imd* expression abolished or severely reduced *drosomycin* activity (Fig. 3A and B). In contrast to Imd pathway members, no member of the Toll pathway tested was able to significantly knock down the *drs-GFP* signal, not even simultaneous knock down of both *dif* and *dorsal*. It therefore appears that the Imd pathway was responsible for the observed AMP expression capable of conferring *S. aureus* resistance.

4. Discussion

The finding that overexpression of *imd* in immune response tissues was capable of conferring resistance to the Gram-positive *S. aureus* was surprising considering traditional roles for the Imd and Toll pathways; the Imd pathway has been thought to be activated in response to Gram-negative bacteria while the Toll pathway was responsible for immunity to Gram-positive bacteria and fungi. Recently this view of *Drosophila* innate immunity has begun to change with numerous findings contradicting traditional views [25–28,30,45,46]. Certainly, this study provides further evidence for a modified view of innate immunity even considering the inherent weakness of overexpression studies.

While phagocytosis likely plays a role in the resistance of *imd* overexpressing flies, humoral defense in the form of AMP activity was found to be sufficient for its resistance as indicated by phagocytic blockage experiments (Fig. 1A). This AMP activity included a traditional Toll pathway reporter, *drosomycin*. While this AMP has been shown to be controlled by both pathways in a tissue dependent manner [44] we observed *drs* expression in tissues shown previously to be controlled by the Toll pathway. It was therefore somewhat surprising to see *imd* expression capable of activating its expression and for this expression to be dependent entirely on the Imd pathway. Again, this may be an artifact of *imd* overexpression, however, further study is warranted to determine the extent to which *imd* can control AMP activity.

It would be a vast oversimplification to speculate that the Imd pathway entirely controls humoral responses considering that it has been shown that both pathways working synergistically are required for proper induction of AMP expression [29]. Furthermore, full AMP expression can occur only upon both phagocytosis, itself dependent on both pathways, and digestion of microbes [5,47]. Therefore, it is likely a combination of both pathways controlling pulses of cellular and humoral responses to specific microbial invaders that leads to a successful defense.

Acknowledgments

We are grateful to J.E. Reichhart, I. Ando, B. Lemaitre, J. Hofmann, Y. Engstrom, T. Aigaki, M. Galko, Kyoto *Drosophila* Genetic Research Center, NIG-Fly Stock Center, Vienna *Drosophila* RNAi Center, and Bloomington Stock Center for fly strains, and D.S. Schneider, H. Hamamoto, and K. Sekimizu for bacterial strains. We are also grateful to H. Aonuma, E. Kuranaga, T. Teramoto, and S. Gururaja Rao for valuable discussions. This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, Culture and Technology to H.K. and S.F., and the Funding Program for Next Generation World-Leading Researchers (NEXT Program) to H.K.

References

[1] J.A. Hoffmann, The immune response of Drosophila, Nature 426 (2003) 33–38.
[2] C.A. Brennan, K.V. Anderson, Drosophila: the genetics of innate immune recognition and response, Annu. Rev. Immunol. 22 (2004) 457–483.
[3] T. Yano, S. Mita, H. Ohmori, Y. Oshima, Y. Fujimoto, R. Ueda, H. Takada, W.E. Goldman, K. Fukase, N. Silverman, T. Yoshimori, S. Kurata, Autophagic control of listeria through intracellular innate immune recognition in drosophila, Nat. Immunol. 9 (2008) 908–916.
[4] E.R. Haine, Y. Moret, M.T. Siva-Jothy, J. Rolff, Antimicrobial defense and persistent infection in insects, Science 322 (2008) 1257–1259.
[5] C.A. Brennan, J.R. Delaney, D.S. Schneider, K.V. Anderson, Psidin is required in Drosophila blood cells for both phagocytic degradation and immune activation of the fat body, Curr. Biol. 17 (2007) 67–72.
[6] B. Lemaitre, M. Meister, S. Govind, P. Georgel, R. Steward, et al., Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila, EMBO J. 14 (1995) 536–545.

[7] B. Lemaitre, E. Nicolas, L. Michaut, J.M. Reichhart, J.A. Hoffmann, The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults, Cell 86 (1996) 973–983.
[8] E.A. Levashina, E. Langley, C. Green, D. Gubb, M. Ashburner, et al., Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*, Science 285 (1999) 1917–1919.
[9] T. Horng, R. Medzhitov, *Drosophila* MyD88 is an adapter in the Toll signaling pathway, Proc. Natl. Acad. Sci. USA 98 (2001) 12654–12658.
[10] P. Ligoxygakis, N. Pelte, J.A. Hoffmann, J. Reichhart, Activation of *Drosophila* Toll during fungal infection by a blood serine protease, Science 297 (2002) 114–116.
[11] Y.T. Ip, M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, et al., Dif, a dorsal-related gene that mediates an immune response in Drosophila, Cell 75 (1993) 753–763.
[12] E. Nicolas, J.M. Reichhart, J.A. Hoffmann, B. Lemaitre, In vivo regulation of the IkappaB homologue cactus during the immune response of Drosophila, J. Biol. Chem. 273 (1998) 10463–10469.
[13] S. Tauszig-Delamasure, H. Bilak, M. Capovilla, J.A. Hoffmann, J.L. Imler, *Drosophila* MyD88 is required for the response to fungal and Gram-positive bacterial infections, Nat. Immunol. 3 (2002) 91–97.
[14] B. Lemaitre, E. Kromer-Metzger, L. Michaut, E. Nicolas, M. Meister, et al., A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense, Proc. Natl. Acad. Sci. USA 92 (1995) 9465–9469.
[15] S. Hu, X. Yang, dFADD, a novel death domain-containing adapter protein for the *Drosophila* caspase DREDD, J. Biol. Chem. 275 (2000) 30761–30764.
[16] S. Rutschmann, A.C. Jung, R. Zhou, N. Silverman, J.A. Hoffmann, et al., Role of Drosophila IKK gamma in a toll-independent antibacterial immune response, Nat. Immunol. 1 (2000) 342–347.
[17] N. Silverman, R. Zhou, S. Stoven, N. Pandey, D. Hultmark, et al., A Drosophila IkappaB kinase complex required for relish cleavage and antibacterial immunity, Genes Dev. 14 (2000) 2461–2471.
[18] S. Stoven, I. Ando, L. Kadalayil, Y. Engstrom, D. Hultmark, Activation of the Drosophila NF-kappaB factor Relish by rapid endoproteolytic cleavage, EMBO Rep. 1 (2000) 347–352.
[19] P. Georgel, S. Naitza, C. Kappler, D. Ferrandon, D. Zachary, et al., Drosophila immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis, Dev. Cell 1 (2001) 503–514.
[20] Y. Lu, L.P. Wu, K.V. Anderson, The Antibacterial arm of the *Drosophila* innate immune response requires an IkappaB Kinase, Genes Dev. 15 (2001) 104–110.
[21] S. Vidal, R.S. Khush, F. Leulier, P. Tzou, M. Nakamura, et al., Mutations in the *Drosophila* dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NFkappaB-dependent innate immune responses, Genes Dev. 15 (2001) 1900–1912.
[22] F. Leulier, S. Vidal, K. Saigo, R. Ueda, B. Lemaitre, Inducible expression of double-stranded RNA reveals a role for dFADD in the regulation of the antibacterial response in *Drosophila* adults, Curr. Biol. 12 (2002) 996–1000.
[23] D. Hultmark, Drosophila immunity: paths and patterns, Curr. Opin. Immunol. 15 (2003) 12–19.
[24] M.S. Dionne, D.S. Schneider, Models of infectious diseases in the fruit fly *Drosophila melanogaster*, Dis. Model. Mech. 1 (2008) 43–49.
[25] M. Hedengren, B. Asling, M.S. Dushay, I. Ando, S. Ekengren, et al., Relish, a central factor in the control of humoral but not cellular immunity in Drosophila, Mol. Cell 4 (1999) 827–837.
[26] L.N. Pham, M.S. Dionne, M. Shirasu-Hiza, D.S. Schneider, A specific primed immune response in Drosophila is dependent on phagocytes, PLoS Pathog. 3 (2007) e26.
[27] E. De Gregorio, P.T. Spellman, P. Tzou, G.M. Rubin, B. Lemaitre, The Toll and Imd pathways are the major regulators of the immune response in Drosophila, EMBO J. 21 (2002) 2568–2579.
[28] S. Rutschmann, A. Kilinc, D. Ferrandon, Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in Drosophila, J. Immunol. 168 (2002) 1542–1546.
[29] T. Tanji, X. Hu, A.N. Weber, Y.T. Ip, Toll and IMD pathways synergistically activate an innate immune response in Drosophila melanogaster, Mol. Cell. Biol. 27 (2007) 4578–4588.
[30] M. Hedengren-Olcott, M.C. Olcott, D.T. Mooney, S. Ekengren, B.L. Geller, et al., Differential activation of the NF-kappaB-like factors Relish and Dif in Drosophila melanogaster by fungi and Gram-positive bacteria, J. Biol. Chem. 279 (2004) 21121–21127.
[31] A. Takehana, T. Katsuyama, T. Yano, Y. Oshima, H. Takada, et al., Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in Drosophila larvae, Proc. Natl. Acad. Sci. USA 99 (2002) 13705–13710.
[32] F. Leulier, C. Parquet, S. Pili-Floury, J.H. Ryu, M. Caroff, et al., The Drosophila immune system detects bacteria through specific peptidoglycan recognition, Nat. Immunol. 4 (2003) 478–484.
[33] S. Pili-Floury, F. Leulier, K. Takahashi, K. Saigo, E. Samain, et al., In vivo RNA interference analysis reveals an unexpected role for GNBP1 in the defense against Gram-positive bacterial infection in Drosophila adults, J. Biol. Chem. 279 (2004) 12848–12853.
[34] P. Leone, V. Bischoff, C. Kellenberger, C. Hetru, J. Royet, et al., Crystal structure of *Drosophila* PGRP-SD suggests binding to DAP-type but not lysine-type peptidoglycan, Mol. Immunol. 45 (2008) 2521–2530.
[35] G. Toba, T. Ohsako, N. Miyata, T. Ohtsuka, K.H. Seong, et al., The gene search system. A method for efficient detection and rapid molecular identification of genes in Drosophila melanogaster, Genetics 151 (1999) 725–737.
[36] H. Hamamoto, K. Kurokawa, C. Kaito, K. Kamura, I. Manitra Razanajatovo, et al., Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms, Antimicrob. Agents Chemother. 48 (2004) 774–779.
[37] M. Elrod-Erickson, S. Mishra, D. Schneider, Interactions between the cellular and humoral immune responses in Drosophila, Curr. Biol. 10 (2000) 781–784.
[38] E. De Gregorio, S.J. Han, W.J. Lee, M.J. Baek, T. Osaki, et al., An immune-responsive serpin regulates the melanization cascade in Drosophila, Dev. Cell 3 (2002) 581–592.
[39] K. Okado, N. Shinzawa, H. Aonuma, B. Nelson, S. Fukumoto, et al., Rapid recruitment of innate immunity regulates variation intracellular pathogen resistance in Drosophila, Biochem. Biophys. Res. Commun. 379 (2009) 6–10.
[40] M. Stofanko, S.Y. Kwon, P. Badenhorst, A misexpression screen to identify regulators of *Drosophila* larval hemocyte development, Genetics 180 (2008) 253–267.
[41] J.R. Huh, I. Foe, I. Muro, C.H. Chen, J.H. Seol, et al., The Drosophila inhibitor of apoptosis (IAP) DIAP2 is dispensable for cell survival, required for the innate immune response to gram-negative bacterial infection, and can be negatively regulated by the reaper/hid/grim family of IAP-binding apoptosis inducers, J. Biol. Chem. 282 (2007) 2056–2068.
[42] J.S. Ayres, D.S. Schneider, A signaling protease required for melanization in Drosophila affects resistance and tolerance of infections, PLoS Biol. 6 (2008) 2764–2773.
[43] N. Shinzawa, B. Nelson, H. Aonuma, K. Okado, S. Fukumoto, M. Miura, H. Kanuka, P38 MAPK-dependent phagocytic encapsulation confers infection tolerance in *Drosophila*, Cell Host Microbe 6 (2009) 244–252.
[44] P. Tzou, S. Ohresser, D. Ferrandon, M. Capovilla, J.M. Reichhart, et al., Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia, Immunity 13 (2000) 737–748.
[45] G.W. Lau, B.C. Goumnerov, C.L. Walendziewicz, J. Hewitson, W. Xiao, et al., The Drosophila melanogaster toll pathway participates in resistance to infection by the gram-negative human pathogen Pseudomonas aeruginosa, Infect. Immun. 71 (2003) 4059–4066.
[46] B.E. Mansfield, M.S. Dionne, D.S. Schneider, N.E. Freitag, Exploration of host-pathogen interactions using Listeria monocytogenes and Drosophila melanogaster, Cell. Microbiol. 5 (2003) 901–911.
[47] B. Charroux, J. Royet, Elimination of plasmatocytes by targeted apoptosis reveals their orle in multiple aspects of the Drosophila immune response, Proc. Natl. Acad. Sci. USA 106 (2009) 9797–9802.