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Contributions of immune responses to developmental resistance in *Lymantria dispar* challenged with baculovirus

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ABSTRACT

How the innate immune system functions to defend insects from viruses is an emerging field of study. We examined the impact of melanized encapsulation, a component of innate immunity that integrates both cellular and humoral immune responses, on the success of the baculovirus *Lymantria dispar* multiple nucleocapsid nucleopolyhedrovirus (LdMNPV) in its host *L. dispar*. *L. dispar* exhibits midgut-based and systemic, age-dependent resistance to LdMNPV within the fourth instar; the LD_{50} in newly molted larvae is approximately 18-fold lower than in mid-instar larvae (48–72 h post-molt). We examined the role of the immune system in systemic resistance by measuring differences in hemocyte immunoresponsiveness to foreign targets, hemolymph phenoloxidase (PO) and FAD-glucose dehydrogenase (GLD) activities, and melanization of infected tissue culture cells. Mid-instar larvae showed a higher degree of hemocyte immunoresponsiveness, greater potential PO activity (pro-PO) at the time the virus is escaping the midgut to enter the hemocoel (72 h post-inoculation), greater GLD activity, and more targeted melanization of infected tissue, which correlate with reduced viral success in the host. These findings support the hypothesis that innate immune responses can play an important role in anti-viral defenses against baculoviruses and that the success of these defenses can be age-dependent.

1. Introduction

Innate immunity in insects is a complex system involving specific responses to a wide variety of pathogens and threats. Understanding the role different components play in pathogen defense is often complex as many aspects of innate immunity are interrelated. The traditional division between cellular and humoral responses is becoming increasingly blurred as new discoveries highlight the strong connections between hemocytes, anti-microbial compounds and enzymes such as prophenoloxidase (pro-PO) (Lavine and Strand, 2002; Cerenius and Soderhall, 2004; Yu and Kanost, 2004; Strand, 2008).

The factors contributing to the melanized encapsulation response are of particular interest because it exemplifies the integration of cellular and humoral responses. Melanized encapsulation is a well-documented response to both wounding and the presence of multicellular pathogens (Schmidt et al., 2001; Lavine and Strand, 2002; Theopold et al., 2004; Strand, 2008), but its role as an anti-viral defense is less well studied, particularly in susceptible hosts outside of model organisms like *Drosophila* spp.

Some resistant or semi-susceptible lepidopterans infected with the insect-specific baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) limit viral spread by cellular encapsulation and melanization of NPV-infected host tissues (Washburn et al., 2000; Trudeau et al., 2001). NPV's initiate infection when a susceptible host ingests occlusion bodies (OBs), which release occlusion derived virions (ODV) that infect the midgut epithelial cells (Pritchett et al., 1984). Infected midgut cells produce budded virions (BV) that escape the midgut by infecting tracheal epidermal cells, which penetrate the basal lamina, and spread infection throughout the host (Volkman, 2007). Infection also spreads via infected hemocytes (Engelhard et al., 1994). Late in the virus life cycle, infected cells produce new ODV that are packaged into occlusion bodies (Riegel and Slavicek, 1997), which are released into the environment upon the host's death. Although the potential for host interference is present at many stages within viral pathogenesis, encapsulation is not usually considered an important anti-viral defense in susceptible lepidopteran hosts because most do not show systemic resistance to viral infection; anti-viral defense in these hosts appears to be primarily restricted to blocking initial infections with midgut-based barriers (Haas-Stapleton et al., 2003).

This is not the case in larvae of the gypsy moth, *Lymantria dispar*, which is permissive to the baculovirus *L. dispar* MNPV (LdMNPV) (Woods and Elkinton, 1987; Barber et al., 1993), but shows age-dependent differences in susceptibility. Insects inoculated orally

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(the natural route of infection) exhibit markedly reduced mortality when inoculated in the middle of the instar as opposed to inoculation within the first 12 h following the molt (Hoover et al., 2002; Grove and Hoover, 2007). In these insects, midgut-based barriers to infection (e.g. midgut cell sloughing) are the greatest contributors to reduced susceptibility, but systemic barriers such as encapsulation or apoptosis of infected tracheae and hemocytes further contribute to limiting viral spread through the host (McNeil, 2008). Interestingly, intrahemocoelically inoculated third and fourth instar larvae also exhibit age-dependent differences in susceptibility (Hoover et al., 2002; Grove and Hoover, 2007), which we suggest is likely true of all instars, although this has not been specifically investigated. Because intrahemocoelic inoculation bypasses midgut-based barriers to infection, this finding indicates that there are age-dependent differences in mortality resulting from differences in systemic barriers to infection, such as cellular encapsulation or apoptosis of infected tissue.

We hypothesize that the mechanism(s) of systemic developmental resistance of *L. dispar* involves immune responses of the host to virally infected tissue. To test this hypothesis, we first determined if immunosuppression of the host through injection of polydnavirus would eliminate systemic developmental resistance. Polydnaviruses (PDV) are obligate, segmented dsDNA viruses associated with many braconid and ichneumonid wasps and are well known disruptors of cellular and humoral immune responses in lepidopterans (Shelby and Webb, 1999). We then compared *in vitro* immunoresponsiveness to foreign targets by hemocytes from newly molted (4^0) and mid-fourth instar larvae (4^{48}-48 h post-molt), both with and without baculovirus challenge.

We also compared phenoloxidase (PO) and FAD-glucose dehydrogenase (GLD) activities in the hemolymph of 4^0 and 4^{48} larvae inoculated with baculovirus. PO and GLD are components of the humoral immune response associated with melanized encapsulation (Lovallo and Cox-Foster, 1999; Nappi and Christensen, 2005). Phenoloxidase has potent anti-microbial properties, and its activation is tightly controlled within the hemolymph to prevent non-specific activation from harming the host (Jiravanichpaisal et al., 2006). PO activity in the hemolymph of *Heliothis virescens* larvae has been shown to have virucidal effects *in vitro* (Shelby and Popham, 2006) and previous research has supported the role of PO and cellular immunity in immune defenses against viruses (Stanley and Shapiro, 2007, 2009; Shrestha and Kim, 2008). GLD is induced during encapsulation responses to pathogens (Cox-Foster and Stehr, 1994; Lee et al., 2005). GLD is involved in the production of free radicals derived from quinones during melanization in insects and has been hypothesized to strengthen melanized capsules (Cox-Foster and Stehr, 1994), which could also negatively impact viral success.

For these experiments, we asked if there were age related differences in PO and GLD activities, and if these differences correlated with hemocyte response to foreign targets and viral mortality. Finally, we compared encapsulation and melanization of infected gypsy-moth tissue-culture cells inserted into 4^0 and 4^{48} larvae to determine if these responses impacted viral success.

2. Methods

2.1. Insect rearing

Gypsy moth eggs were obtained from the USDA (Otis ANGB, MA) and reared on artificial diet (Southland gypsy moth diet, Southland Insect Diet, Lake Village, AR) until they became premolts to the fourth instar, indicated by head capsule slippage, as described by Hoover et al. (2002). Premolts were collected and stored at 28 °C until they molted to the fourth instar. After molting, larvae were either placed on artificial diet in individual plastic cups (29.6 mL, WNA, Chelmsford, MA) and fed for 48 h before being used in experiments (designated as 4^{48} larvae), or used immediately after molting (designated as 4^0 larvae).

2.2. Virus preparation

For all experiments, we used the LdMNPV pol + LacZ7H5 strain of baculovirus; this construct, hereafter referred to as LdMNPV-7H5, expresses *lacZ* from *Escherichia coli* driven by the hsp70 promoter of *Drosophila* (McNeil, 2008); under this promoter, *lacZ* expression is constituative. BV of LdMNPV-7H5 was obtained by collecting cell-free culture medium of infected Ld652Y cells provided by Dr. Jim Slavicek (USDA, Delaware, OH) suspended in TnMFH + 9%FBS (Atlanta Biologicals, Atlanta, GA). BV was stored in 500 μL aliquots at −80 °C until needed. Occlusion bodies (OBs) were isolated from infected *L. dispar* larvae and stored in PBS at 4 °C as described previously (Hoover et al., 2002).

2.3. Immunosuppression of L. dispar

L. dispar larvae were immunosuppressed using a crude extract of polydnavirus (PDV) obtained from the ovaries and calyces of adult female *Glyptapanteles flavicoxis* wasps. Extracts of these tissues contain a high concentration of PDV particles and have been used as an immunosuppressant in conjunction with baculovirus inoculation in previous studies (Beckage et al., 1994; Washburn et al., 2000).

Cocoons or adult wasps were obtained from the USDA (Beneficial Insects Research Lab, Newark, DE or the Invasive Insect Biocontrol and Behavior Laboratory, Beltsville, MD). Cocoons were kept in cardboard cups (0.24 L, Sweetheart Cup Company Inc., Owings Mills, MD) with a moist sponge at 25 °C and a 14:10 (L:D) photoperiod and checked daily for adult emergence. Adults were stored at 12 °C in cardboard cups with a sponge moistened with water and another sponge soaked in honey for up to 7 d or until needed for experiments. Wasps were placed at room temperature the day before an experiment was conducted.

The ovaries and calyces of female wasps were removed and stored in 500 μL of TnmFH + 9%FBS insect cell medium. The tissue was homogenized and filtered to isolate PDV, according to the protocol of Lovallo and Cox-Foster (1999). The ovary/calyx extract was diluted with TnmFH + 9%FBS to yield a concentration of 0.33 wasp equiv./μL, held at 4 °C, and used within 6 h.

The impact of immunosuppression on systemic developmental resistance to baculovirus was assessed by injecting 4^0 and 4^{48} larvae intrahemocoelically with 0.33 wasp equiv. of ovary/calyx extract and 3.7 $TCID_{50}$ of LdMNPV-7H5 BV using a disposable, tuberculin syringe with a 32-gauge sharp needle and a micro-injector (Pax 100, Burkard Scientific, Uxbridge, Middlesex, UK). Larvae were inoculated first with PDV (1 μL) and then immediately after with baculovirus (1 μL). Injections were done by orienting the larva perpendicular to the syringe, inserting the needle at the base of the third proleg for PDV and the second proleg for BV, administering the dose, and waiting 12–20 s before removing the needle to ensure all the inoculum entered the larva. Ten to 15 insects in each experiment were injected with only PDV or TnmFH + 9%FBS insect cell medium as controls. After being inoculated, insects were placed in individual plastic cups, fed on artificial diet *ad libitum*, maintained at 25 °C with a 18:6 (L:D) photoperiod, and checked daily until pupation or death.

2.4. Hemocyte behavior in response to an immune stimulus

Age-related immunoresponsiveness of hemocytes was compared using an *in vitro* protocol adapted from Lovallo and Cox-Foster (1999). Briefly, a four-well tissue culture chamber (Nalge Nunc

International, Rochester, NY) was prepared by coating each well with a layer of agarose (Seaplaque low melting point agarose, Cambrex, Rockland, ME). A 10 μL drop of agarose mixed with either the immuno-elictor laminarin (0.125%, w:v, Sigma Chemical Co., St. Louis, MO) or insect cell media (TnMFH + 9%FBS) was added to the center of each well. The entire tissue chamber was stored at 4 °C to solidify the droplet. To observe hemocyte behavior, the tissue chamber was placed on the stage of an Axiovert 405M microscope, and 490 μL of TnMFH + 9% FBS was added to a single well. Then a 10 μL hemolymph sample from either a 4^0 or 4^{48} larva was added to the well and the hemocytes were allowed to settle for 1–3 min. Hemolymph samples were collected by surface sterilizing the larvae in 70% ethanol for ~30 s, drying them a on a paper towel, and clipping a proleg with a pair of sterile dissecting scissors. The hemolymph was dripped onto parafilm on ice. To determine if hemocytes would respond directly to free BV in the media (as opposed to virus infected tissue), a 1 μL aliquot of LdMNPV-7H5 BV (3.7×10^4 $TCID_{50}$/mL) or TnmFH + 9%FBS in 60% glycerol (carrier for BV at 1:1, v/v) was added to the well directly over the agarose droplet immediately after adding the hemolymph. To assess the influence of PDV on hemocytes responses, some treatments contained a 1 μL aliquot (0.33 wasp equiv.) of the crude PDV extract from *G. flavicoxis*, extracted and prepared as described above.

For each sample, a 200× still image of the edge of the agarose droplet with the highest concentration of hemocytes present was taken after 20 min using Scion Image (v.1.63) software. For each age group, images were taken of hemocytes in separate wells responding to a laminarin droplet and an insect medium droplet, both with and without BV present. The images were analyzed for encapsulation, as indicated by flattened, clumped cells at the margin of the droplet. The percent coverage of the droplet margin with encapsulation was calculated for each image using ImageJ software (v. 1.36b). These assays were repeated a minimum of five times for each treatment within an age group, with the exception of the glycerol + BV + PDV and insect medium droplet treatment, which was repeated twice.

2.5. Humoral defense activities in host hemolymph

We measured the activities of PO and GLD in hemolymph from 4^0 and 4^{48} larvae. Larvae from both cohorts were inoculated orally with an LD_{50} of LdMNPV-7H5 occlusion bodies in 60% glycerol (165 OB/μL for 4^0 larvae and 4000 OB/μL for 4^{48} larvae to obtain similar mortality levels). Two types of controls of each age group were prepared: one group was mock-inoculated with 60% glycerol only and the other group was untreated. Inoculations were performed with a syringe and a 30-gauge, blunt needle. The needle was inserted into the mouth and 1 μL of inoculum was delivered into the anterior midgut using a microinjector, as described above for intrahemocoelic inoculation. After inoculation, the larvae were kept in individual plastic cups and maintained at 25 °C with a 18:6 h (L:D) photoperiod. Hemolymph samples were collected at 36, 48, and 72 h post-inoculation (hpi) from 8 to 10 larvae of each age from viral, mock, and uninoculated control treatments. These time points were chosen to reflect the time period in LdMNPV pathogenesis when infection is beginning to escape the midgut through the tracheal system and into the hemolymph (McNeil, 2008), and thus is when humoral defenses are most likely to play a role in anti-viral responses. For all samples, 10 μL of hemolymph were diluted in 60 μL of Grace's insect medium (Lonza, Walkersville, MD) in the well of a 96-well plate (Cellstar, Greiner Bio-one, Monroe, NC) and gently agitated for ~3 min. To measure baseline PO activity, 10 μL of a hemolymph dilution was mixed with 10 μL of de-ionized water and 200 μL of 0.2 M L-3-(3,4-dihydroxyphenyl)alanine (L-DOPA, TCI America, Portland, OR) in 0.1 M phosphate buffer and the absorbance was read at 490 nm for 20 min using a Spectramax 250 spectrophotometer (GMI Inc., Ramsey, MN). Potential (total activatable) PO activity was measured by adding 10 μL of hemolymph dilution to 10 μL of 10% cetylpyridinium chloride (CPC, MP Biomedicals Inc., Solon, OH) (Hall et al., 1995) and 200 μL of 0.2 M L-DOPA; absorbance was measured at 490 nm as described above.

Because baseline phenoloxidase (PO) and total activatable PO (pro-PO) activities in the hemolymph were highly variable among larvae within a treatment group and age, we calculated the ratio of total activatable PO to baseline PO in the hemolymph to obtain a more reliable comparison across treatments (Fig. 3). A higher value of this ratio indicates greater reserves of unactivated PO (pro-PO) present in the hemolymph. Pro-PO is difficult to measure directly but has been demonstrated to be negative regulator of PO activity and melanin formation (Kan et al., 2008).

GLD activity was determined by mixing 10 μL of diluted hemolymph with 200 μL of 2,6-dichlorophenolindophenol (DCIP) reagent (0.1 M Tris–HCl pH 7.0, 76 mM β-D-glucose, 48 μM DCIP) and measuring absorbance at 600 nm for 20 min (Lovallo and Cox-Foster, 1999). Protein concentration of each hemolymph sample was determined using a modified Bradford assay (Cox-Foster and Stehr, 1994; Lovallo and Cox-Foster, 1999). Specific enzyme activity for PO and GLD was expressed as enzyme activity per mg of protein in each sample.

To confirm infection in virally inoculated larvae after collecting a hemolymph sample, each insect cadaver was processed for *LacZ* expression as described previously (Plymale et al., 2008). The cadavers were scored for the presence of infected cells (LacZ signaling) in the midgut and tracheae. This experiment was replicated twice.

2.6. In vivo encapsulation response to agarose implants containing infected tissue culture cells

To examine melanized encapsulation of infected cells, pyrogen-free implants containing infected Ld652Y cells in agarose plugs were prepared and inserted into 4^0 and 4^{48} larvae. Cells were grown in Excell 420 + 5% serum + insect cell medium (SAFC Biosciences, Lenexa, KS) to a concentration of $\sim 1.0 \times 10^5$ cells/mL. A 10 mL aliquot of the cells was inoculated with 500 μL of LdMNPV-7H5 BV (3.7×10^4 $TCID_{50}$/mL) and incubated at 27 °C for 5 d. Another 10 mL aliquot of cells was incubated at 27 °C for the same amount of time. The cells were prepared for tissue implants by centrifuging 3 mL of each cell suspension for 2 min at 1000 × *g*. The supernatant was discarded and 750 μL of sterile, pyrogen-free

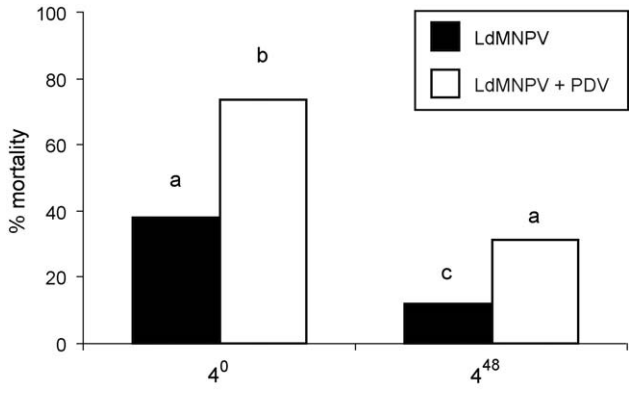

Fig. 1. Effect of immunosuppression by polydnavirus (PDV) on LdMNPV mortality in larvae inoculated intrahemocoelically immediately after molting to the fourth instar (4^0) or 48 h after molting to the fourth instar (4^{48}). Significantly different values are represented by different letters over the bar (nominal logistic regression; $p < 0.05$). Error bars = 1SE. Bars represent mean percentage mortality of 3 replicates of the experiment; $N = 20$ larvae per treatment in each replicate.

2% (w:v) agarose (Seaplaque low melting point agarose) were added. An additional treatment of 50 μL of LdMNPV-7H5 BV only (3.7×10^4 $TCID_{50}$/mL) and 750 μL of agarose was also prepared along with a control treatment of agarose alone. The treatments were gently vortexed and 750 μL of each were aliquoted into separate wells of a 24-well tissue culture plate. The plate was stored at 4 °C for up to 12 h, or until needed.

Tissue implants were delivered into 4^0 and 4^{48} larvae using sterile glass micro-capillary tubes (10–20 μL, Drummond Scientific Co., Broomall, PA) pulled into needles by hand over an open flame, with a needle bore of ~0.11 mm. Treatments were loaded into separate micro-capillary needles by pressing the blunt end of the needle into the solidified agarose repeatedly until the tube was filled with agarose. The loaded needles were connected to a plastic syringe and tissue implants were injected by inserting the tip of the needle into the base of the second proleg and injecting 1–1.5 μL of material. Larvae were starved for 4 h prior to implantation to allow food to pass through the midgut and reduce the chances of puncturing the gut. Just prior to implantation, larvae were surface sterilized with 70% ethanol. A new needle was used between treatments and/or after every 15th larva was injected.

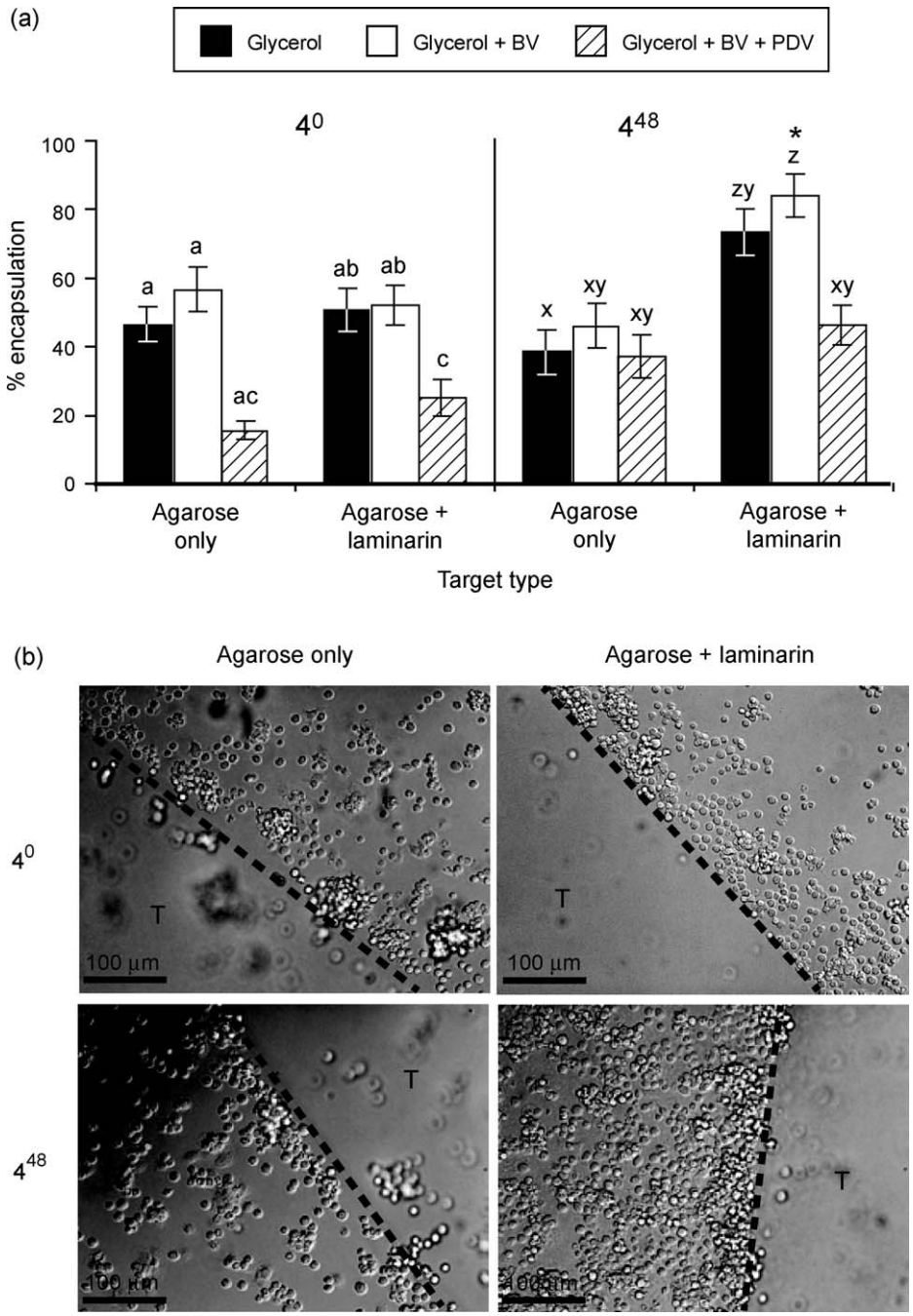

Fig. 2. Hemocyte immunoresponsiveness to a laminarin target as a function of larval age post-molt the fourth instar. (a) Percent area of agarose targets encapsulated with hemocytes from 4^0 and 4^{48} larvae with glycerol in the media (black bars), glycerol and BV (3.7×10^4 $TCID_{50}$/mL) (white bars), or glycerol, BV, and PDV (hashed bars). Significant differences within a larval age group are indicated by different letters over the bar; significantly greater values between 4^0 and 4^{48} larvae within a treatment are indicated by an asterisk over the bar (ANOVA of arcsine transformed data; $p < 0.05$). Error bars = 1SE; N = 2–8 larvae for each treatment. (b) Typical images show the hemocytes' association with the margin (dotted line) of the target (T). Scale bar = 100 μm.

2.7. Measuring melanization and LacZ signaling of tissue culture implants

At 24, 48, and 72 h post-implantation, 10 larvae from each treatment were dissected. These time points were chosen based on the reported infection cycle of LdMNPV (Riegel and Slavicek, 1997) and based on evidence that baculovirus infection in some lepidopteran larvae affects phenoloxidase (PO) levels during the first 72 h following inoculation (Sparks et al., 2008), which could be related to melanization ability. An additional 5–15 larvae from each treatment were observed for 21 d post-implantation to determine if final viral mortality, initiated by the infected implants, differed between age groups. Larvae that showed signs of septicemia were discarded, typically 5–10 larvae per age group and treatment in each replicate. After dissection, the tissue implants, which were found as flattened, irregular clusters of agarose, were removed and placed into separate wells of a 96-well tissue culture treated plate (Nalge Nunc International, Rochester, NY) with 100 μL of 4% (w:v) paraformaldehyde in cytoskeleton extraction buffer (10 mM PIPES, 60 mM sucrose, 100 mM potassium chloride, 5 mM magnesium acetate, 1 mM EG). The side of the implant with the largest surface area was photographed with a digital camera (Nikon Coolpix 950) at 40× using a dissecting microscope and lit with external fiber-optic lamps.

The degree of melanization was determined for each implant according to a method adapted from Bailey and Zuk (2008). Briefly, each image was converted to an 8-bit grayscale format using ImageJ analysis software (v. 1.6.0_05). The implant was outlined

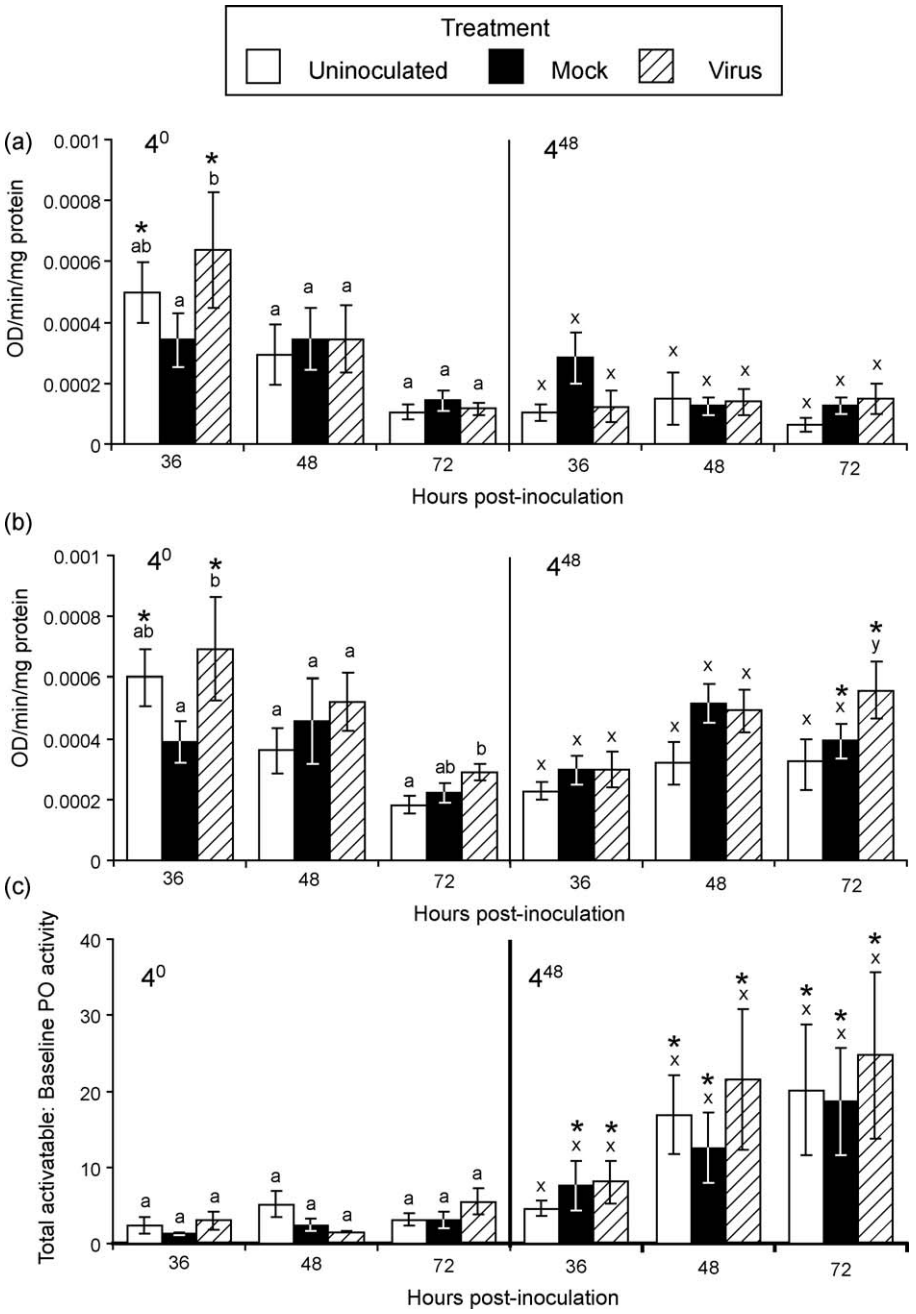

Fig. 3. Phenoloxidase (PO) activities in 4^0 and 4^{48} larvae. (a) Baseline PO activity, (b) total activatable PO activity (pro-phenoloxidase), and (c) their ratio (activatable:baseline) were measured for uninoculated larvae (white bars), glycerol inoculated larvae (black bars), and virally inoculated larvae (hashed bars). Different letters over the bar indicate significantly different values for a given age group within a time point post-inoculation; significantly greater activities between 4^0 and 4^{48} larvae within a treatment at each time point are indicated by an asterisk over the bar (Kruskal–Wallis test, $p < 0.05$). Error bars = 1SE; N = 10–20 larvae for each treatment per time point.

using the incorporated "Freehand selections" tool, copied to a new image file, and the total area of the implant was measured using the incorporated "Measure" tool. Then the melanized portion of the implant was delineated using the "Threshold" tool to select the portion of the image with a grayscale threshold value below 140, which corresponds to melanization. Using the "Measure" tool again, the total area of the image was measured along with the proportion of that total selected by the "Threshold" tool. The melanized proportion of the implant was then determined using the following formula:

$$\%\,\text{of implant melanized} = \frac{\%\,\text{of image with threshold value below 140}}{\text{total area of implant}/\text{total area of image}}$$

After incubating at room temperature for 30 min, the paraformaldehyde solution was removed from the implants and replaced with 75 μL of 0.08% (w:v) X-gal to detect cells infected with the virus. The plates were incubated at room temperature overnight in the dark. The next day, the X-gal solution was removed and 100 μL of 4% paraformaldehyde solution was added to each well. Again, the implants were photographed under constant lighting conditions at 40× using a dissecting microscope.

The level of LacZ signaling for each implant was determined using a method similar to that used for measuring melanization. The "Threshold Colour" plugin written for ImageJ (Gabriel Landini, University of Birmingham, England, UK, http://www.dentistry.bham.ac.uk/landinig/software/software.html) was used to select the portions of the image (HSB format) with a hue of 139–146, which isolated portions of the implant with LacZ signaling. These selections were converted to grayscale and the area of the implant signaling LacZ was determined as described above. This method was sensitive enough to detect single foci of infection (~10 μm in diameter) on the implants.

To confirm viral infection in larvae from which the implants were collected, the insect cadavers were fixed in 4% paraformaldehyde for ~24 h. The cadavers were rinsed twice using cytoskeleton extraction buffer and then immersed in 0.08% X-gal solution for 24 h. The X-gal was removed and the cadavers were scored for the presence of infected cells among the hemocytes, tracheal epidermis, and fat body. The cadavers were also scored for the presence of melanized encapsulations on the tracheae. This experiment was repeated three times.

2.8. Statistical analyses

Mortality data from the immunosuppression bioassay were analyzed using nominal logistic regression. Data from the hemocyte immunoresponsiveness experiments and for the melanization and LacZ signaling in implants were arcsine transformed and analyzed by ANOVA and *post hoc* Tukey's HSD. The data for PO and GLD activities of hemolymph samples were not normally distributed and were analyzed using the Kruskal–Wallis test. Differences in the progression of baculovirus infection in larvae implanted with tissue culture cells, the presence of encapsulation on tracheae, and final viral mortality between age groups were analyzed using Chi-square contingency analysis. Differences were deemed significant with a p-value < 0.05. All statistics were performed using JMP statistical software (v. 8.0, SAS Institute).

3. Results

3.1. Effects of immunosuppression on systemic developmental resistance

Co-inoculation of 4^0 and 4^{48} *L. dispar* larvae with baculovirus and PDV significantly increased mortality by baculovirus in both age groups by over 2-fold (Fig. 1). Moreover, immunosuppression of 4^{48} larvae produced baculovirus mortality that was equivalent to that of 4^0 larvae inoculated with baculovirus only. There was no mortality in larvae inoculated with PDV only or with insect cell media (data not shown).

3.2. Hemocyte behavior in response to an immune stimulus

Hemocytes collected from 4^{48} larvae had a markedly stronger response to the immuno-elicitor laminarin compared with controls (Fig. 2a). The hemocytes from 4^{48} larvae encapsulated a larger proportion of an agarose droplet containing laminarin compared with an agarose-only droplet and accumulated in greater abundance at the margin of the droplet containing laminarin (Fig. 2b).

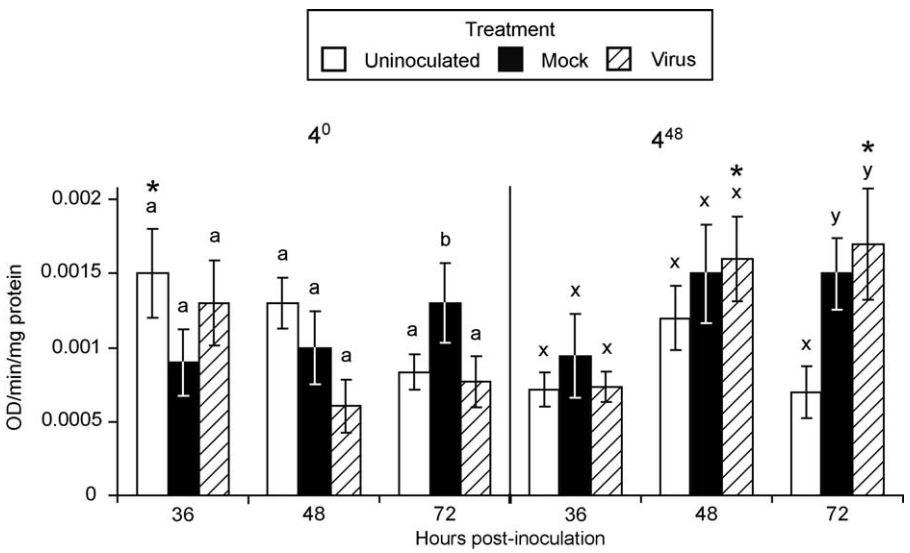

Fig. 4. FAD-glucose-dehydrogenase (GLD) activity in 4^0 and 4^{48} larvae. Bars represent the GLD activity in hemolymph samples of uninoculated larvae (white bars), mock inoculated larvae (black bars) or virus inoculated larvae (hashed bars). Different letters over the bar indicate significantly different values within an age group at each time point post-inoculation; significantly greater values between 4^0 and 4^{48} larvae within a treatment at each time point are indicated by an asterisk over the bar (Kruskal–Wallis test, $p < 0.05$). Error bars = 1SE; N = 10–20 larvae for each treatment per time point.

Hemocyte responses from 4^0 larvae were indistinguishable from the responses of hemocytes to control targets.

The presence of free budded virus added to the media containing hemocytes did not alter the response of hemocytes to the laminarin targets. Regardless of larval age, hemocytes did not respond to the target in the presence of free BV any differently than they did to treatments without BV (Fig. 2a). If PDV was added to the medium along with budded virus plus glycerol, however, the hemocytes from both 4^0 and 4^{48} larvae responded significantly less to laminarin targets by almost 50% (Fig. 2a).

3.3. Humoral defense activities in host hemolymph

The ratio of total activatable PO (pro-PO) to baseline PO in the hemolymph was higher in 4^{48} larvae than 4^0 larvae for almost all treatments at all time points, indicating there were significantly greater reserves of pro-PO in 4^{48} larvae than in 4^0 larvae (Fig. 3). In 4^{48} larvae at 72 h post-inoculation, when the virus is moving from the tracheae into the hemolymph (McNeil, 2008), there was also a significant increase in the pro-PO in virally inoculated larvae compared to the other treatments. Activated PO was higher in the hemolymph of 4^0 larvae than the hemolymph of 4^{48} larvae at early time points.

GLD activity in virally inoculated 4^{48} larvae was significantly higher than in 4^0 larvae at 48 and 72 hpi (Fig. 4). Although the GLD activity in virally inoculated 4^{48} larvae did not differ from that of mock inoculated larvae, at 72 hpi activities in both mock and virally inoculated treatments were significantly higher than that of uninoculated controls. There were no consistent significant differences among treatment groups in 4^0 larvae; GLD activity in mock larvae was higher than virally inoculated larvae at 72 hpi, but there were no other significant differences (Fig. 4).

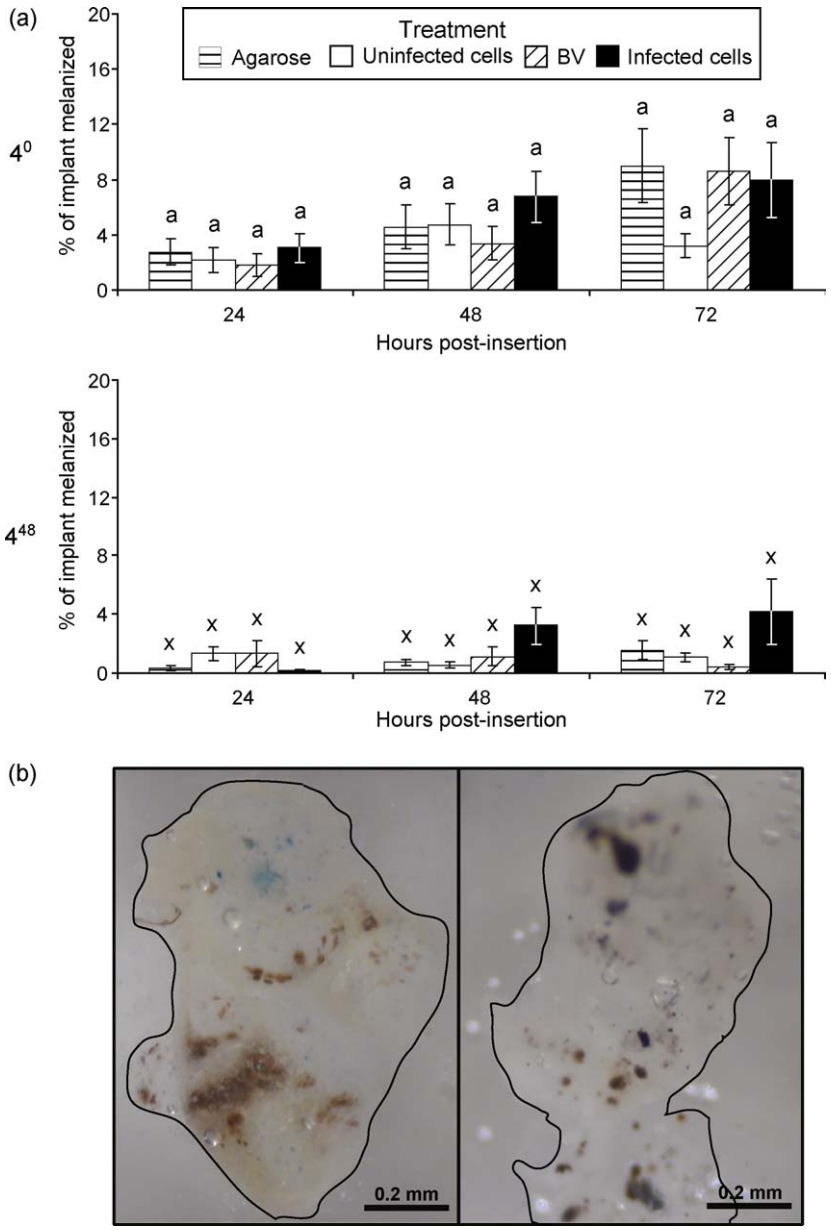

Fig. 5. Melanization of agarose implants removed from 4^0 or 4^{48} larvae at 24, 48 or 72 h post-insertion. (a) Implants contained agarose only, uninfected Ld652Y cells, free BV within the agarose, or infected Ld652Y cells. Different letters over the bar indicate significant differences within an age group and time point (ANOVA of arcsine transformed data; $p < 0.05$). Error bars = 1SE; N = 5–20 larvae per treatment group and time point. Typical melanization pattern for implants containing infected cell culture cells removed from (b, left) 4^0 larvae and (b, right) 4^{48} larvae at 48 h post-implantation. Blue color indicates LacZ signaling (infected) cells. Scale bar = 0.2 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Despite inoculation of 4^{48} larvae with a viral dose that was 24-fold higher than for 4^0 larvae, there were no significant differences between the age groups in final mortality (4^0 larvae: 45%; 4^{48} larvae: 23%; Fisher's Exact test $p = 0.06$) or between the proportions of insect cadavers signaling LacZ at any time point post-infection (data not shown).

3.4. In vivo encapsulation response to agarose implants containing infected tissue culture cells

In 4^0 larvae, hemocytes tended to respond more broadly to implants than in 4^{48} larvae. The percentage of the implant that was melanized tended to appear greater in those removed from 4^0 larvae than 4^{48} larvae in all treatments (1.3–20 times greater), although there were no statistically significant differences between the two larval ages at any time point (24 h post-implantation: $F_{(7,\ 113)} = 1.4$, $p = 0.21$; 48 h post-implantation: $F_{(7,\ 92)} = 1.4$, $p = 0.20$; 72 h post-implantation: $F_{(7,\ 91)} = 1.6$, $p = 0.16$) (Fig. 5a). There were no significant differences among treatments for either 4^0 or 4^{48} larvae, but within 4^{48} larvae there was a trend for greater melanization in implants containing infected cells at 48 and 72 h post-implantation. Within 4^0 larvae the percent melanization tended to increase over time for all treatments except for implants with uninfected cells.

Despite the lack of differences in total melanization of the implants between the two ages of larvae, the melanization patterns were markedly different. The melanized regions of implants from 4^{48} larvae appeared to be more targeted to the infected cells (signaling lacZ) and better defined than those on implants from 4^0 larvae (example shown in Fig. 5b).

3.5. LacZ signaling of implants

There were significant differences in LacZ signaling between treatments at all time points post-implantation (24 h post-implantation: $F_{(7,\ 93)} = 7.2$, $p < 0.0001$; 48 h post-implantation: $F_{(7,\ 77)} = 5.0$, $p = 0.0001$; 72 h post-implantation: $F_{(7,\ 65)} = 13.9$, $p < 0.0001$) (Fig. 6). LacZ signaling in implants containing infected tissue culture cells inserted into 4^0 larvae was significantly higher than in agarose only treatments at 24 h post-implantation and higher than all other treatments (agarose only, uninfected cells, or free BV) at 48 and 72 h post-implantation. Most importantly, it was higher than that of implants from 4^{48} larvae at 72 h post-implantation (Fig. 6). In 4^{48} larvae, LacZ signaling was greater in implants containing infected tissue culture cells than other treatments at 24 h post-implantation, but for the remaining time points, the amount of LacZ signaling did not differ from controls, and was never higher than signaling in 4^0 larvae (Fig. 6).

3.6. LacZ signaling and encapsulation in larvae implanted with infected cells

Viral infection spread from the tissue culture infected implants into the larval tissues in both ages of larvae. Infection in larvae implanted with infected cells was first observed in the hemocytes and tracheal epidermal cells, as evidenced by LacZ signaling in dissected larvae. In 4^0 larvae, infection continued to spread into the tracheal epidermal cells and fat body until, by 72 h post-implantation, all larvae signaling LacZ had infections in most tissues (Fig. 7).

Infections in 4^{48} larvae from infected tissue culture implants began similarly in the hemocytes but without any tracheal

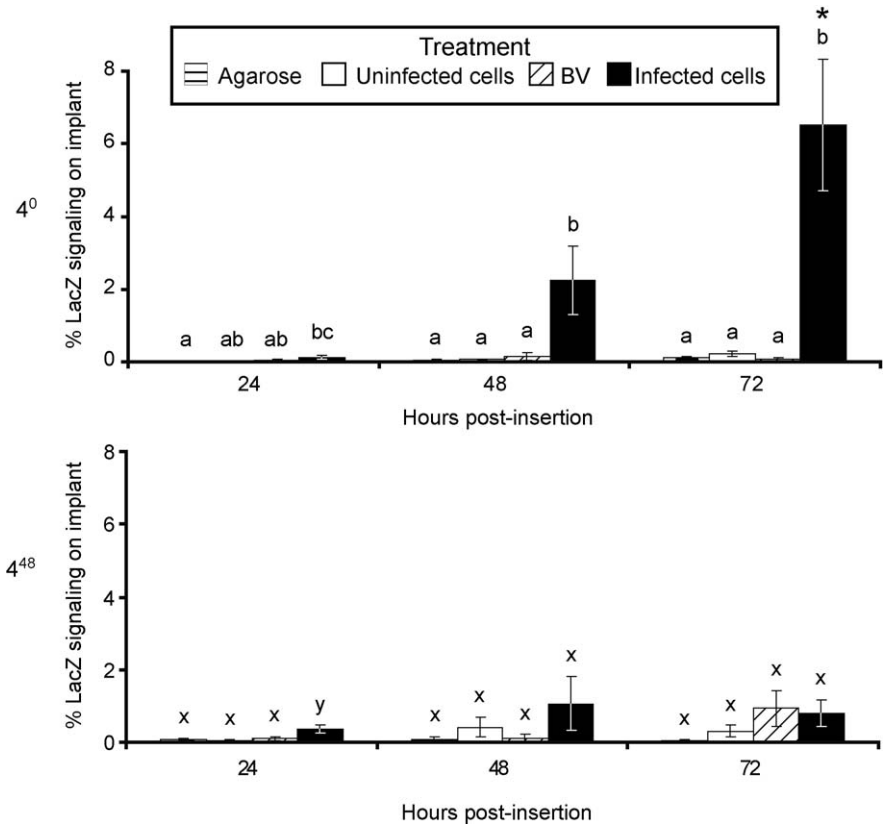

Fig. 6. Proportion of LacZ signaling in agarose implants removed from 4^0 and 4^{48} larvae for all treatments at 24, 48, or 72 h post-insertion. Implants contained agarose only, uninfected Ld652Y cells, naked BV within the agarose, or infected Ld652Y cells. Different letters over the bar indicate significant differences within an age group at each time point; significantly greater values between 4^0 and 4^{48} larvae within a treatment at each time point are indicated by an asterisk over the bar (ANOVA of arcsine transformed data, $p < 0.05$). Error bars = 1SE; $N = 4$–18 larvae per treatment group per time point.

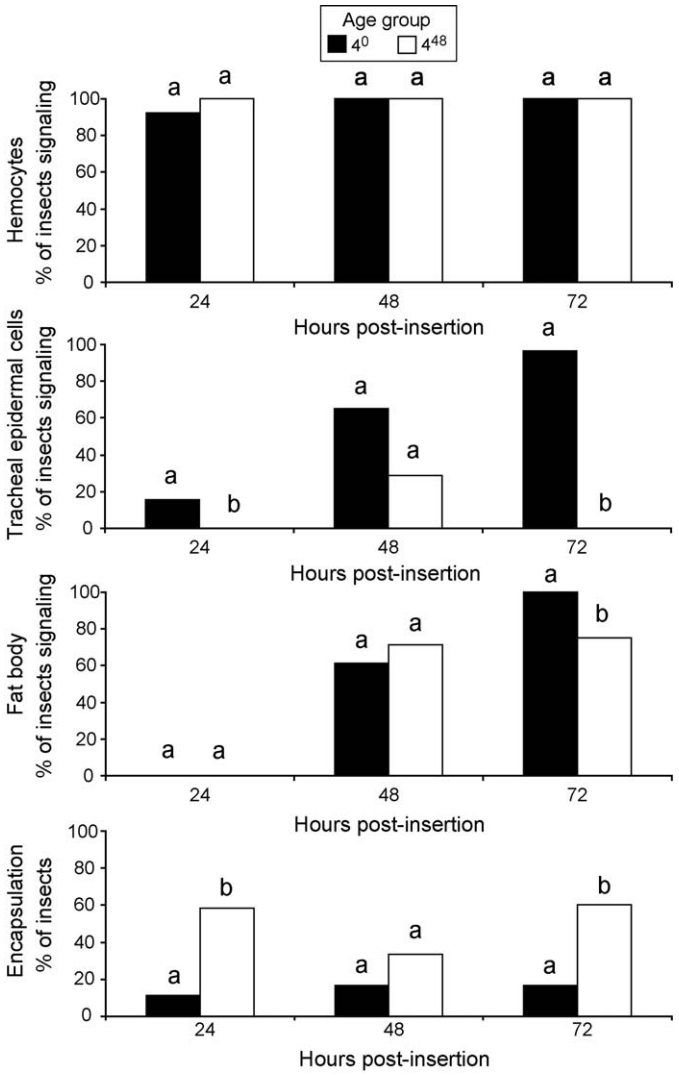

Fig. 7. Proportion of 4^0 and 4^{48} cadavers implanted with infected Ld652Y cells signaling LacZ in different tissue types at 24, 48, or 72 h post-insertion. Proportion of all larvae with encapsulated tracheal epidermal cells is also shown for both age groups. Different letters over the bar indicate significant differences between age groups within a time point and tissue type (Chi-square test, $p < 0.05$). $N = 2$–29 larvae.

epidermal or fat-body cell infections. At 48 h post-implantation, infections were found in all three tissues, although hemocyte infections were most prevalent (Fig. 7). By 72 h post-implantation, there were no tracheal epidermal cell infections detected in implanted 4^{48} larvae, and the incidence of fat body infections was significantly lower compared to 4^0 larvae ($\chi^2_{(1,\,35)} = 6.6$, $p = 0.01$). Incidence of encapsulation of tracheae was greater in 4^{48} larvae than in 4^0 larvae at all time points post-implantation, although the difference was significant at 24 and 72 h, but not 48 h, post-implantation (Fig. 7).

LacZ signaling in tracheal trunk junctions appeared greater in 4^0 larvae than in 4^{48} larvae by 72 h post-implantation (Fig. 8). LacZ signaling in this tissue type tended to be more prevalent with time in 4^0 larvae, while in 4^{48} larvae, it increased in prevalence for the first 48 h but was reduced by 72 h post-implantation.

In the subset of larvae with implants that were observed for 21 d post-implantation, there were no viral deaths in 4^0 or 4^{48} larvae implanted with agarose only or uninfected cells and only one 4^0 larva implanted with free BV only died from virus. There was extensive viral death in 4^0 and 4^{48} larvae implanted with infected cells, but mortality was significantly greater in 4^0 larvae than in 4^{48} larvae (100% vs. 57%, respectively).

4. Discussion

Systemic resistance of *L. dispar* to LdMNPV is strongly influenced by immune responses in the host, as indicated by the large increase in viral mortality when the immune system was disrupted by PDV. Additionally, the fact that viral mortality in immunosuppressed mid-instar larvae was equal to that of non-immunosuppressed newly molted larvae indicates that immune responses play an important role in systemic developmental resistance. These data suggest several factors that could explain why non-immunosuppressed mid-instar larvae are less susceptible to LdMNPV, such as increased immunoresponsiveness of the hemocytes (Fig. 2). The profound reduction in immunoresponsiveness to the laminarin targets in the presence of PDV coupled with the reduction in viral mortality when PDV is co-inoculated with LdMNPV indicates hemocyte mobilization is a key component of anti-viral defense, but the fact that hemocytes did not respond directly to free BV in the medium suggests this response is directed at infected cells, not the virus itself. The greater reserves of pro-PO in the hemolymph (Fig. 3), higher GLD activities in response to immune challenge (Fig. 4), and more targeted melanization of infected tissue (Fig. 5) suggest the hemocyte response is also enhanced by the humoral components of immunity. The pro-PO:activated PO ratio is a useful approach for estimating the amount of unactivated PO present in the hemolymph and the reserves of the zymogen that potentially could be utilized for an additional immune response. Increased reserves of pro-PO may be indicative of an induced response as the insect begins to mount anti-viral defenses and represents increased transcription and translation of pro-PO (Yang and Cox-Foster, 2005; Eleftherianos et al., 2008).

Most importantly, our data indicate that immune responses in mid-instar larvae significantly reduced viral success and slowed viral spread through the host. There is evidence of age-related differences in immune responses to other pathogens. The hemocytes of *Manduca sexta* have a similar differential response to an immune stimulus (bacteria) with larval age; hemocytes from older larvae formed more nodules than those from younger larvae of the same instar (Eleftherianos et al., 2008). Another study found that the hemocytes of *Drosophila* were activated by the presence of tissue infected with the RNA virus Drosophila X and responded in a more targeted fashion (Zambon et al., 2005), similar to our observation of a more targeted melanization response to infected tissue culture cell implants in mid-instar *L. dispar* (Fig. 5).

Reduced LacZ signaling in the tracheae of mid-instar insects indicates that in addition to encapsulating infected tissue, the developmentally resistant host was able to eliminate established infections. Previous work with *L. dispar* challenged with LdMNPV demonstrated that apoptosis occurs in infected tracheal epidermal cells and hemocytes (McNeil, 2008). Apoptosis may thus be a mechanism for eliminating tracheal epidermal cell infections (Fig. 7) and limiting LacZ signaling in the tracheal trunk branches (Fig. 8) of mid-instar insects. It is likely these processes contributed to the reduced viral mortality of mid-instar larvae. Rivkin et al. (2006) reported that tracheal trunk junctions are important foci of infection early in the pathogenesis of *Spodoptera littoralis* larvae intrahemocoelically inoculated with AcMNPV, and it appears the same is true in the *L. dispar*/LdMNPV system. Tracheal infections have been shown to be the primary route of viral spread in many baculovirus/lepidopteran systems (Engelhard et al., 1994), so limiting tracheal infections is a powerful mechanism for reducing viral success.

While many of the immune responses involved in systemic developmental resistance are present in both age groups of larvae, in most cases they were more active in mid-instar larvae than in newly molted larvae. Developmental resistance to LdMNPV within

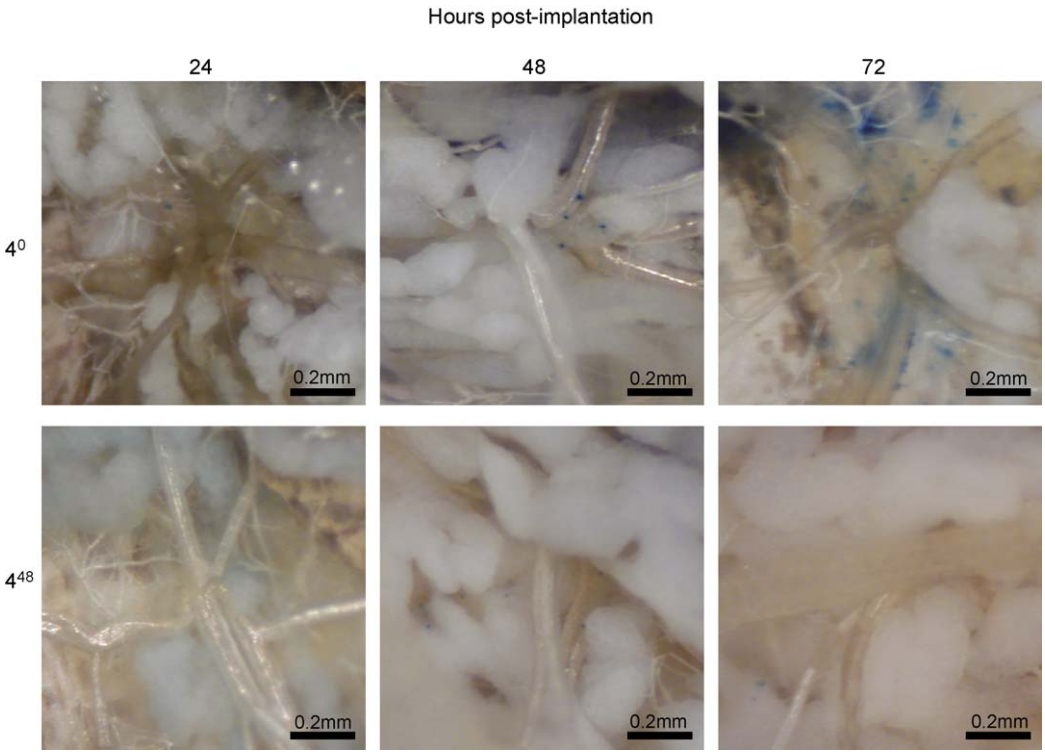

Fig. 8. LacZ signaling in the tracheal junctions of larvae implanted with infected Ld652Y cells embedded in agarose. Dark blue tissues indicate viral infection (LacZ signaling). Images were collected from larvae at 24, 48, and 72 h post-implantation and represent typical levels of infection. Note increasing LacZ signals in 4^0 larvae and decreasing signals in 4^{48} larvae over time. Scale bars = 0.2 mm. N = 6–19 larvae at each time point per age group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

both the third and fourth instar of *L. dispar* begins between 12 and 24 hpi, increasing further between 48 and 72 hpi, with some return to susceptibility near the end of the instar as larvae begin to premolt to the next instar (Hoover et al., 2002; Grove and Hoover, 2007). Thus, evidence to date indicates that anti-viral immune responses are closely linked with the host's life cycle. In nature, LdMNPV infects *L. dispar* larvae orally, but larvae do not begin feeding until 12–24 h post-molt, so targeted defenses against baculovirus infection are not required early in the instar, particularly given that immune responses are costly (Brown, 2003). However, before their cuticle sclerotizes, early instar larvae are more susceptible to physical external threats, such as wounding and attack by parasitoids. The generalized melanization response to agarose implants that we observed in 4^0 larvae and the generally higher levels of activated PO in their hemolymph is consistent with the physiological ability to deal with these external threats as a newly molted larva. Rapid, generalized PO activation has been implicated in wound healing in insects (Hall et al., 1995; Cerenius and Soderhall, 2004) and in defense against parasitoid eggs and larvae (Lovallo et al., 2002; Cerenius and Soderhall, 2004; Hodgetts and O'Keefe, 2006).

In contrast, 4^{48} larvae are actively feeding and must defend against the increased threat of oral pathogens like LdMNPV while maintaining defenses against other threats. Our evidence of a greater ability to target immune responses may indicate a mechanism for balancing these defense needs with the limited energy and nutrients available to the host. Mid-instar larvae mobilize hemocytes more aggressively and direct melanization to infected tissues more effectively than newly molted larvae. The increase in the reserves of unactivated PO in 4^{48} larvae at 72 hpi could indicate specific induction in response to the virus because that is the time when virions are leaving the midgut and attempting to enter the hemocoel. The overall increase in unactivated PO we observed in 4^{48} larvae could also reflect changes in hemolymph chemistry as the larva prepares for the next molt. The increased GLD activity in mid-instar larvae also indicates a more targeted immune response. Increased GLD activity is associated with killing reactions in melanized encapsulations and is a tightly regulated response (Lovallo and Cox-Foster, 1999), which makes GLD more effective as a component of the targeted defense of mid-instar larvae than the generalized response of newly molted larvae.

The baculovirus could also be acting to disrupt host defenses, which could contribute to developmental resistance. The reduction in activated PO in 4^0 larvae at 72 h post-inoculation could be the result of the virus disrupting or eliminating hemocytes, which could allow the virus to spread. Viral infection appears to be eliminated in some tissues, but many baculoviruses also contain genes which inhibit apoptosis in infected host tissues. LdMNPV contains two inhibitor of apoptosis genes, *iap2* and *iap3* (Kuzio et al., 1999), and, although their functionality in the host is unresolved, they could play a role in how the virus is able to overcome and persist in the presence of substantial host defenses.

Understanding the implications and mechanisms of anti-viral immune responses in *L. dispar* provides more evidence that innate immune responses, which are conserved among many animal groups, are complex and effective against a wide variety of pathogens. Determining the mechanisms of systemic developmental resistance may also provide valuable information for modeling the population dynamics of *L. dispar*. The United States Forest Service produces LdMNPV as the biopesticide Gypchek (Podgwaite, 1999). Manipulating the factors controlling developmental resistance, possibly by incorporating immunosuppressive agents, could eventually help make Gypchek a more effective, targeted, biological control agent against an important forest pest.

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