Functional Regulatory Divergence of the Innate Immune System in Interspecific *Drosophila* Hybrids

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Abstract

In order to investigate divergence of immune regulation among *Drosophila* species, we have engaged in a study of innate immune function in F1 hybrids of *Drosophila melanogaster* and *D. simulans*. If pathways have diverged between the species such that incompatibilities have arisen between interacting components of the immune network, we expect the hybrids to display dysregulation of immune genes. We have quantified gene induction in hybrid and parental flies in response to bacterial infection. These results show that although the hybrids do not suffer widespread immune breakdown, they show significantly different regulation of many immune genes relative to the parents. We examine this divergence in terms of additivity and expression differences among genes, observing distinct patterns of dysregulation among functional groups within the pathways of the innate immune system. The functional groups most sensitive to misexpression in the hybrids are the downstream components of the network, indicative of some propagation of dysregulation throughout the immune pathways. Interestingly, this dysregulation does not appear to associate with phenotypic differences in bacterial load after infection in hybrids, possibly highlighting some robustness of function of the innate immune response to perturbations like hybridization.

Key words: *Drosophila*, innate immunity, hybrid dysregulation, misexpression, functional divergence.

Introduction

Innate immunity has garnered interest in numerous areas of biological research due to its ubiquity throughout the animal kingdom. In addition to serving as the only response to infection in invertebrates, the innate immune system provides an initial, generalized attack against invading microbes and activates the adaptive immune response in humans and other vertebrates (Medzhitov and Janeway 1997). The deep evolutionary conservation of these pathways across taxa underscores the importance of maintaining their components and functions for effective immune response. Nevertheless, divergence in the innate immune response is evident—even between closely related species—in the form of nucleotide or amino acid sequence differences among orthologs, as well as through changes in the collection of genes and gene families that comprise the immune pathways (Date et al. 1998; Sackton et al. 2007). This may reflect distinct pathogen environments, driving diverse selection pressures and different roles of immune response in the context of varying life history traits among species (Schmid-Hempel 2003).

Drosophila has become an effective model system for investigating the evolution of innate immunity due to the well-studied genetics of insect response to infection and the comparative resources available. Although immune genes as a group may undergo greater levels of positive selection than nonimmune genes in flies (Schlenke and Begun 2003; Obbard et al. 2009), it has also become evident that different functional subgroups of immune genes show distinct patterns of evolution. For example, antimicrobial peptides (AMPs), despite facing direct contact with invading microbial cells, have shown relatively few amino acid substitutions among *Drosophila* species (Clark and Wang 1997; Date et al. 1998; Ramos-Onsins and Aguadé 1998; Lazzaro and Clark 2003; Jiggins and Kim 2005). On the other hand, some immune-related recognition proteins (scavenger receptors) show evidence of rapid evolution (Lazzaro 2005). More recently, large-scale examination of patterns of selection acting upon each of the immune genes in the genomes of multiple *Drosophila* species has become possible. Sackton et al. (2007) used the sequenced genomes of six species in the *melanogaster* subgroup to make sweeping comparisons of patterns of selection among different functional classes of immune genes. In addition to the wealth of sequence data available for the genomes of these *Drosophila* species, the use of genome-wide expression arrays has allowed for investigation into the details of the regulation of immune response in flies. Numerous studies using these arrays before and after microbial infection (De Gregorio et al. 2001; Irving et al. 2001; Apidianakis et al. 2005) have clarified the dynamics of immune response, solidifying existing models of innate immunity as well as identifying new genes and pathways that are regulated in response to infection.

Throughout an organism, there are numerous systems whose regulatory components may coevolve (e.g., Dover 1992; Ludwig et al. 2000; Shaw et al. 2002). While maintaining proper regulation within a species, if the interactions have diverged separately in two lineages, then incompatibilities

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may arise in interspecific hybrids (True and Haag 2001; Landry et al. 2007). Divergence between closely related species has been inferred from irregular development or enzyme expression patterns in interspecific hybrids (Whitt et al. 1973; Whitt et al. 1977; Dickinson et al. 1984; Parker et al. 1985), and more recently, regulatory divergence between species has been quantified by measuring genome-wide expression levels in F1 hybrids using microarrays (e.g., Michalak and Noor 2003; Ranz et al. 2004; Auger et al. 2005; Moehring et al. 2007; Mavarez et al. 2009). These studies have revealed numerous instances of nonadditivity of expression in hybrid individuals relative to parental phenotypes, indicative of disruption of evolved regulatory mechanisms. Furthermore, in complex regulatory networks, one would expect hybrids to be particularly prone to dysregulation; if one or more portions of a pathway have diverged between parental species such that they result in incompatibilities in the hybrids, these may propagate throughout the network, manifesting in large-scale disruptions of regulatory phenotypes.

The balance of both conserved features and rapidly evolving elements within a coordinated set of pathways makes the *Drosophila* innate immune system particularly interesting in terms of network evolution. A complex network may be especially prone to regulatory disruption in the context of hybrids, where numerous small deviations in the structure or control of the pathway may result in decreased network efficacy. Because the innate immune pathways contain many interacting components and regulatory elements, it is likely that these have diverged between species and that interspecific hybrids may bear phenotypes reflecting this divergence. Indeed, immune-related genes have previously been found to be overrepresented among genes overexpressed in hybrids between *Drosophila melanogaster* and *D. simulans* (Ranz et al. 2004), indicative of constitutive misexpression of these genes within hybrids, and reflective of the rapid evolution of immune gene regulation between these species, relative to genome-wide patterns of divergence.

To investigate the evolution of regulatory elements of the innate immune system and their responses to infection in *Drosophila*, we have quantified dysregulation of the immune response in interspecific hybrids by assaying transcript abundance in *D. melanogaster*, *D. simulans*, and their F1 hybrids before and after infection. If genes throughout the innate immune pathways have diverged between these two species, we expect the hybrids to display nonadditive levels of expression and expression change after bacterial infection. Additionally, if different portions of these networks bear different levels of interspecific regulatory divergence, the patterns of dysregulation throughout the hybrid immune response should reflect those differences.

Materials and Methods

Fly Lines and Crosses

Inbred stocks of *D. melanogaster zygotic hybrid rescue* strain (provided by A. Orr) and a Tsimbazaza strain of *D. simulans* (provided by H. Hollocher) were used to construct hybrid crosses. Lines of each were maintained in laboratory cultures, and from these, we collected *D. melanogaster* virgin females and *D. simulans* males. Interspecific crosses were set up with approximately 10 *D. melanogaster* females and 10 *D. simulans* males per vial. Intraspecific crosses were also set up simultaneously, with about 10 females and 10 males apiece, to produce *D. melanogaster* and *D. simulans* offspring under similar conditions and at similar ages as the F1 hybrid flies. F1 hybrid female flies (male hybrids from this cross are not viable), along with *D. melanogaster* and *D. simulans* female flies, were collected after eclosion.

Bacterial Cultures and Infections

To assay response to bacterial infection in the flies, we infected them with Gram-negative *Serratia marcescens*. This bacterium, chosen based on its previous use for immune studies in *D. melanogaster* (Lazzaro et al. 2004), was derived from ATCC strain 13880. Bacterial cultures for infections were grown overnight from freezer stocks to a concentration of optical density at 600nm (OD_{600}) $\approx$ 1.0. Female offspring from *D. melanogaster*, *D. simulans*, and the *D. melanogaster* $\times$ *D. simulans* hybrid cross were infected at approximately 3–7 days after eclosion. Flies were infected by pricking their thoraces with 0.1-mm tungsten needles (Fine Science Tools, Foster City, CA) dipped in bacterial culture.

Transcript Quantification Using BeadChip Arrays

To estimate expression differences before and after bacterial infection in hybrid flies and those from parental strains, we used custom BeadChip Arrays (Illumina Inc., San Diego, CA) to quantify transcript abundance in the samples. These were designed to include probes for 171 immune-related genes along with 542 genes representing controls or pathways investigated in other experiments (Sackton et al. 2010). See supplementary table S1, Supplementary Material online, for a full list of genes included on the BeadChips, including classification of immune-related genes by functional group. The probes on the BeadChips were designed from the genome sequence of *D. melanogaster*, and although a handful of the 50-bp probes had as many as six or seven bases mismatching with the *D. simulans* sequence, the overall trends observed here did not change when only probes with zero or one mismatches were analyzed (supplementary fig. S1, Supplementary Material online). Note that because the tests of additivity and induction rely on differences in signals from each gene under various conditions, they are not sensitive to potential differential hybridization of the *melanogaster* and *simulans* sequences to the *melanogaster*-based bead oligos. Even though we do not obtain estimates of absolute counts of transcripts of each species, the statistical tests of effects in F1 hybrids remain valid.

Flies from each strain were snap frozen in liquid nitrogen at 6 and at 12 h after infection, in three replicate pools of approximately 12–15 flies each. Uninfected flies were also

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frozen immediately after infection of the others to measure baseline expression levels. For each sample, we isolated messenger RNA using a Trizol:chloroform extraction, and then we synthesized complementary DNA and hybridized it to the BeadChips using the manufacturer's protocol. BeadChips were scanned, and the resulting signal values were normalized across arrays and across chips using qspline in the beadarray R package.

Bacterial Load Quantification

Following previous studies (e.g., Lazzaro et al. 2004), bacterial clearing ability was estimated by quantifying bacterial load in infected flies. Approximately 12 or 25 h after infection, *D. melanogaster*, *D. simulans*, and F1 hybrid flies were homogenized, three at a time, in 500 μl of lysogeny broth (LB). These samples ($n = 4$–9 for each line and time point) were then plated onto agar plates using a spiral plater (Spiral Biotech, Bethesda, MD). Plates were kept overnight to allow colonies to grow enough to be counted by a colony counter to infer bacterial concentration inside each homogenate sample. Plates were visually inspected to ensure that colonies counted showed size and morphology expected.

Statistical Analysis

To test for expression differences before and after bacterial infection, we used mixed linear models incorporating infection status as a fixed effect along with other random effects:

$$y_{ijk} = \mu + \text{Infection}_i + \text{Probe}_j + \text{Replicate}_k + \sum_{ijkl} \quad (1)$$

Here, y is the fluorescence signal indicating transcript abundance, and Infection ($i = 1, 2$) represents the infection status of the flies (either uninfected or infected), included as a fixed effect. Probe ($j = 1, 2$), representing the two separate probes for each gene on the array, and Replicate ($k = 1$–3) were each included as random effects. Transcript abundance is estimated by $\log_2$(Signal), where Signal is the normalized measurement from the arrays, log transformed to achieve a more normal distribution. These tests were performed separately for each individual gene, at both 6 and 12 h after infection, and for *D. melanogaster*, *D. simulans*, and F1 hybrid flies. In order to assess significance of the results of these tests, we compared the coefficients of the infection effect to a null distribution comprised of coefficients calculated from tests of the same model with expression data permuted 1,000 times relative to the genotype and infection status for each gene. These permutation tests directly generate the null distribution of the model and provide an accurate assessment of the P value even in the face of departures from normality. In the end, the parameter test P values and the permutation P values were highly correlated, consistent with the reasonably good fit of the residuals to the normal distribution.

To evaluate differences in expression levels among these groups of flies, we used similar mixed models to test for significant effect of group or "species" on expression. The models were set up as follows:

$$y_{ijk} = \mu + \text{Species}_i + \text{Probe}_j + \text{Replicate}_k + \sum_{ijkl} \quad (2)$$

In this case, y is again a fluorescence signal strength indicating a transcript level, and Species ($i = 1$–3) is a fixed effect including the three groups of flies, *D. melanogaster*, *D. simulans*, and F1 hybrids. Probe and Replicate are both included as random effects as in equation 1. Here, we tested differences among flies for each gene on the chip, examining each treatment type and time point separately. As above, significance for each test was determined by comparison to a null distribution of coefficients calculated using data permuted 1,000 times.

In addition to expression differences among flies, we also quantified induction differences, where induction represents the change in expression before and after infection. To achieve this, we employed similar mixed models, including a term to test the interaction effect of infection status (infected vs. uninfected) on species differences in expression:

$$y_{ijklm} = \mu + \text{Species}_i + \text{Infection}_j + (\text{Species x Infection})_k + \text{Probe}_l + \text{Replicate}_m + \sum_{ijklmn} \quad (3)$$

using the same setup as equation 2, with the addition of Infection ($j = 1, 2$), representing the infection status of the flies, along with an interaction term between this and Species groups as fixed effects. These interactions were examined separately for 6 h after infection and 12 h after infection for each gene individually. Once again, we permuted the expression values 1,000 times and collected coefficients for the interaction term against which we could compare the actual results to determine the significance for each test.

For each gene showing differences in expression or induction among the groups of flies, we tested for the presence of nonadditive expression (or induction) in the F1 hybrids relative to parental levels. The null hypothesis was that F1 expression level was equal to the average of the parental expression values, indicative of entirely additive effects. To test the validity of this hypothesis for each gene, we performed tests using models set up like equation 2, where the only species groups included were parental (*D. melanogaster* and *D. simulans* samples combined) and F1 hybrid flies. Significant differences, again assessed by permutation tests, between parental mean and hybrid expression values allowed us to reject the hypothesis of complete additivity. For these genes, we tested whether hybrid expression showed evidence of dominance or transgressive variation. To test for dominance, the null hypothesis was that F1 expression levels equaled one of the parental expression levels when the two species themselves were significantly different. If the hybrid displayed expression levels outside the range of the parental species and those differences were significant, then these were interpreted as cases of transgressive effects.

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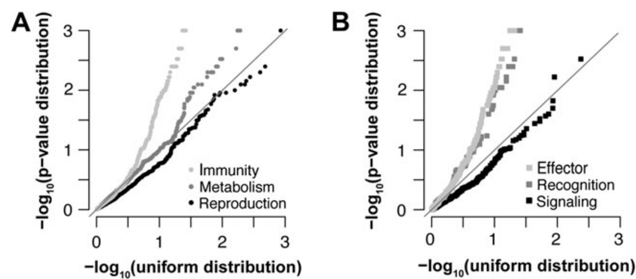

FIG. 1. Distributions of observed *P* values for tests of expression differences before and after infection in groups of genes compared with values from a uniform distribution, as a null expectation. Plot (*A*) shows immunity, metabolism, and reproduction genes. Plot (*B*) displays functional groups within immunity genes: recognition, signaling, and effector genes. Distributions of groups in both (*A* and *B*) vary significantly in the proportion of genes with $P < 0.05$ (χ^2, df = 2, $P = 4.55 \times 10^{-8}$ and $P = 3.50 \times 10^{-18}$, respectively).

To compare induction patterns of genes belonging to the Toll and immune deficiency (imd) pathways in the context of their functional groupings within the pathways, we organized genes by degree of similarity in patterns of expression changes after infection using a simple hierarchical agglomerative clustering algorithm (Cluster 3.0, de Hoon et al. 2004). Genes were grouped with hierarchical, centroid linkage clustering using Euclidean distances to quantify similarity. In addition, we included an expected gene order based on relative location in the known humoral pathways. This informed the clustering such that once nodes of the gene tree were defined, the orientation of each node ordered the genes according to pathway location, wherever possible. When the trees for each group of genes were defined, we used Java TreeView 1.1.3 (Saldanha 2004) to visualize the gene clusters and associated dendrograms. To quantify clustering of gene expression patterns relative to layout of the genes in the Toll and imd pathways, we calculated the correlation coefficients of gene orders from cluster analysis with those in the immune network. To determine significance of each correlation value, we permuted the clustered gene orders 1,000 times and calculated correlations for each to obtain a null distribution, and then we calculated *P* values for the actual correlation coefficients based on these distributions.

Results

Expression Changes after Infection

To examine differences in immune response between hybrid and parental flies, we quantified transcript abundance for genes related to innate immunity and other pathways (including those involved in metabolism and reproduction) using custom Illumina BeadChip arrays. Induction (or repression) levels of genes represented on the arrays were estimated by comparing transcript levels before and after infection. Genes showing significant differences (with a nominal $P < 0.05$) in transcript abundance between uninfected and infected flies (at 6 or 12 h after infection) were considered to be induced or repressed. Out of all immune-related genes on the array, 14.8% of the tests for all three groups of flies (76 out of 513) at 6 h and 8.8% of tests (45 out of 513) at 12 h had $P < 0.05$, both of which include significantly more than the 5% that would be expected by chance (χ^2, degrees of freedom [df] = 1, $P_{6\ \mathrm{h}} = 1.99 \times 10^{-24}$, $P_{12\ \mathrm{h}} = 8.86 \times 10^{-5}$). Because many of these genes were chosen to be on the BeadChip because of their previously observed induction in flies following bacterial infection, this result is entirely expected. Several nonimmune genes on the array also show some expression changes; yet, these are not as substantial, with 8.2% of tests (89 out of 1089) at 6 h and only 1.6% of tests (17 out of 1089) at 12 h bearing significantly different expression levels before and after infection with $P < 0.05$. Distributions of *P* values for tests of expression differences before and after infection are shown in figure 1*A* for the genes whose products function in immunity, metabolism, or reproduction. Observed *P* values are plotted here in order to examine deviations from the null expectation of uniformly distributed values. Genes in these groups bear significantly different distributions of *P* values, with substantially more immune genes having induction tests with $P < 0.05$ than either of the other classes of genes (χ^2, df = 2, $P = 4.55 \times 10^{-8}$). Furthermore, even within the immune group, genes corresponding to different functions also show distinct levels of expression changes after infection (χ^2, df = 2, $P = 3.50 \times 10^{-18}$, fig. 1*B*).

Because numerous investigations have quantified expression differences in *Drosophila* after bacterial infection using whole-genome arrays, we compared our results with data from three of these (De Gregorio et al. 2001; Irving et al. 2001; Apidianakis et al. 2005) to evaluate the uniqueness of the genes induced here. Out of the set of genes showing expression changes in our study, 37.6% (64 out of 170) of the genes with differences at 6 h and 54% (34 out of 63) with differences at 12 h were also found to be induced or repressed after infection in one or more of the other screens (based on the definitions of significant expression differences given in each paper). Similarly, out of the 227 genes on the BeadChips that had been shown to be induced in at least one of these previous studies, 39.6% (90 genes) had significant differences in expression after infection in this experiment. Causes of these discrepancies may include genetic variation among the lines used, differences among *D. melanogaster*, *D. simulans*, and F1 hybrids (although the *melanogaster* samples showed no greater overlap than those from the other flies), and a difference in expression technologies and experimental setups. Furthermore, these studies assayed response to infection with a variety of bacteria; infections with different species of varying levels of pathogenicity in the flies will inevitably have different influences on patterns of expression genome wide.

Patterns of Nonadditivity in F1 Hybrid Expression

In addition to comparing the number of genes changing expression after infection in the three groups of flies, we also examined levels of expression of genes in F1 hybrids relative to those in *D. melanogaster* and *D. simulans*. To quantify regulatory differences between hybrids and

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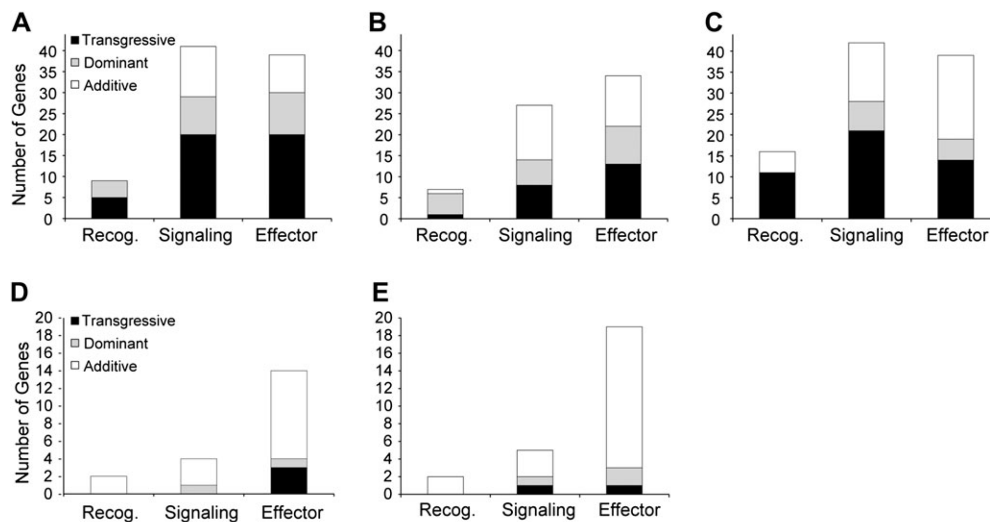

Fig. 2. Number of immune genes differently expressed among *Drosophila melanogaster*, *D. simulans*, and F1 hybrid flies in recognition (Recog.), signaling, and effector classes showing patterns of additive, dominant, or transgressive expression in the F1 hybrids. (*A*) Expression in uninfected flies. (*B*) Expression in flies 6 h after infection with *Serratia marcescens*. (*C*) Expression in flies 12 h after infection with *S. marcescens*. (*D*) Induction/repression in flies 6 h after infection with *S. marcescens*. (*E*) Induction/repression in flies 12 h after infection with *S. marcescens*. *Y* axes for plots represent number of genes differently expressed among groups of flies belonging to each pattern of expression.

parental species in the expression of immune-related genes, we classified all genes with expression or induction differences among fly groups as showing additive, dominant, or transgressive patterns (supplementary table S2, Supplementary Material online). We find a substantial number of immune genes showing nonadditive expression levels in hybrids—in uninfected flies as well as after infection. Interestingly, not only do levels of nonadditivity in immune genes vary across time points, but immune genes in distinct functional roles (recognition, signaling, or effector) also show diverse patterns of additive, dominant, and transgressive expression, as shown in figure 2. Here we observe that expression levels of most of these immune genes—both before and after infection—are nonadditive in F1 hybrids, relative to parental levels. On the other hand, induction/repression levels (expression differences before and after infection) appear to be much more conserved in these flies; fewer genes show differences in levels of expression change among groups of flies, and most genes that are differently induced/repressed show additive effects in hybrids. In fact, at six hours after infection, only genes with effector functions show evidence for transgressive effects of induction or repression; other genes that differ among species groups only display additive or dominant effects.

Expression Levels among Different Functional Classes of Immune Genes in F1 Hybrids

To further examine the differences between hybrid and parental mean expression levels indicated by the nonadditive effects apparent in groups of the immune genes, we plotted expression levels for all immune genes in hybrids against parental mean expression of these genes, shown in figure 3. Through these comparisons, it is evident that a substantial number of immune genes appear as outliers, indicative of nonadditive expression in the hybrids. These outliers (arbitrarily defined as points that lie outside of the 95% confidence interval of the regression line) appear among genes expressed in uninfected flies, as well as in flies 6 or 12 h after infection (fig. 3*A*). Strikingly, the patterns of outliers in expression levels vary widely among functional classes of immune genes. More specifically, genes coding for effectors are highly overrepresented among the high-expression outliers (χ^2, df = 1, $P = 7.19 \times 10^{-13}$), where hybrid expression is higher than parental mean expression, whereas genes coding for signaling proteins are highly overrepresented among low-expression outliers (χ^2, df = 1, $P = 4.03 \times 10^{-11}$), with lower hybrid than parental mean expression.

We also examined levels of change in expression after infection in these flies (fig. 3*B*). Although many genes in hybrids are induced (or repressed) at levels consistent with additivity, there are numerous immune genes that appear as outliers in this comparison. In this case, there are not significant differences between outliers with respect to induction levels, but effector genes are once again significantly overrepresented among all the outliers (χ^2, df = 1, $P = 1.01 \times 10^{-9}$).

Coordinated Regulatory Differences in Hybrid Expression Change after Infection

To evaluate differences in induction or repression of immune genes in hybrids compared with flies from the parental species in the context of defined humoral pathways, we examined the patterns of genes clustered together based on similar patterns of induction/repression across samples (fig. 4). When the order of the clustered genes was compared with the order of gene products within the humoral immune pathways, we found these to be significantly correlated for genes in both the Toll and imd pathways (correlation coefficients = 0.643, 0.689; P = 0.008, 0.017,

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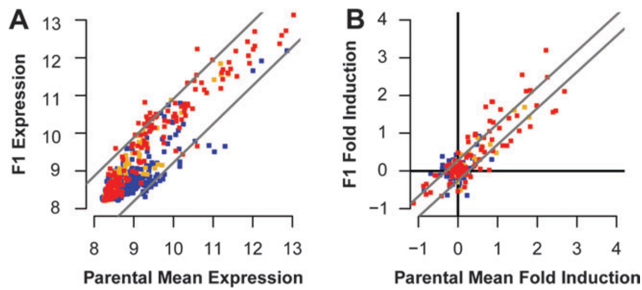

FIG. 3. F1 versus parental mean expression (*A*) and induction/repression (*B*) levels in immune genes. Expression is defined as $\log_2$(Signal), where Signal is an arbitrary measure of fluorescence from the BeadChip. Fold induction is the difference in the expression values before and after infection. Recognition genes are shown in gold, signaling genes in blue, and effectors in red. Gray lines represent 95% confidence intervals of the regression line for the data.

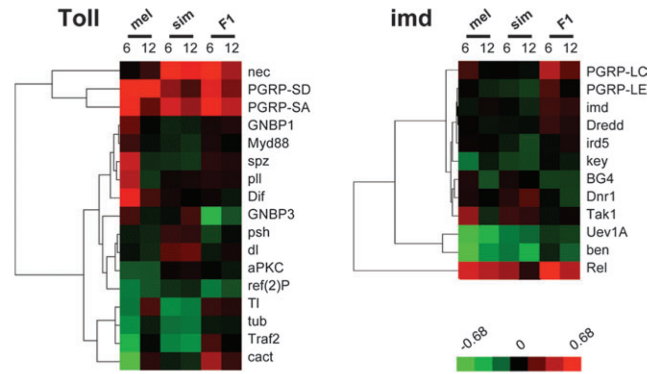

FIG. 4. Genes throughout Toll and imd pathways (excluding effectors) clustered based on patterns of changes in expression levels after infection among samples of *Drosophila melanogaster* (mel), *D. simulans* (sim), and F1 hybrids (F1). Scale bar indicates magnitude of expression change ($\log_2$[Signal] in infected flies—$\log_2$[Signal] in uninfected flies).

respectively). Through this, we see that induction patterns across the *melanogaster*, *simulans*, and F1 groups appear to be more similar among genes closely grouped within the immune pathways.

To examine F1-specific patterns of coordinated gene regulation, we noted which uniquely induced or repressed genes in hybrids belonged to the Toll and imd pathways (fig. 5). Only a handful of genes throughout these pathways showed expression changes only in the hybrids; yet, some patterns in the regulation of these genes are apparent, at least at 6 h after infection. At this time point, the only genes that we observe to be uniquely regulated in the hybrids all belong to the imd pathway and show positive expression changes after infection, consistent with a systemic overinduction of genes in the imd pathway in response to Gram-negative infection in hybrids.

Bacterial Clearing Ability in Hybrids and Parental Species

As a proxy for systemic response to bacterial infection, we quantified bacterial load in terms of colony-forming units per fly, at 12 and 25 h after infection with *S. marcescens* in *D. melanogaster*, *D. simulans*, and F1 hybrid flies. As shown in figure 6, hybrid flies show similar bacterial levels after infection compared with the parental species, with no significant differences in load among groups of flies at 12 h (analysis of variance [ANOVA], $P = 0.9815$) or at 25 h after infection (ANOVA, $P = 0.7719$). Additionally, mortality was observed in the flies with and without bacterial infection; the hybrids showed no significant difference in survival compared with the parental flies, up to 4 days after infection (see supplementary fig. S2, Supplementary Material online) (*t*-test, $P = 0.251$).

Discussion

Here we report our findings of regulatory divergence in innate immunity between *D. melanogaster* and *D. simulans*, inferred from dysregulation of the immune response in the interspecific hybrids of these flies. Despite a general trend of additivity of expression of most immune genes in the hybrids, significant evidence for nonadditive regulation was also detected across diverse parts of the innate immune pathway, indicative of divergent control of expression in response to bacterial infection in the two species. Interestingly, although the hybrids contain many genes that differ from parental levels of expression, the systemic immune phenotypes do not appear to be compromised in the hybrid flies; bacterial levels and survival rates after infection are consistent between hybrid and parental flies, indicating a robustness of the immune response to regulatory perturbations.

It is not surprising that the F1 hybrids examined here display distinctive expression profiles compared with parental species. If regulatory controls of transcription throughout the genome have diverged separately in two species, new combinations of *cis-* and *trans*-regulatory factors that arise in the hybrids may lead to unique expression patterns in the hybrids. Previous studies quantifying genome-wide transcript levels in closely related *Drosophila* species and their hybrids have also found substantial evidence for nonadditive expression levels in interspecific hybrids (Ranz et al. 2004; Graze et al. 2009). Contrasting allele-specific expression in parental species and F1 hybrids has afforded the opportunity to tease apart *cis-* and *trans*-acting factors that result in altered expression (Wittkopp et al. 2004, 2008; Graze et al. 2009), and a consistent picture is emerging that the largest interspecific changes in expression are driven by *cis*-acting divergence but that there remains extensive *trans*-acting polymorphism within species, and in fact, *trans*-acting compensation of *cis*-acting divergence also occurs (Landry et al. 2005). To the extent that *cis*-acting variation impacts only expression of the *cis*-allele, its effects will be more additive, whereas *trans*-acting variation will more likely show a dominant effect on both target alleles. Such differences may have a significant impact on fixation of regulatory variation (Lemos et al. 2008), and the among-gene heterogeneity in apparent additivity seen in F1 hybrids may be a historical consequence of fixation of *cis-* and *trans*-modulators.

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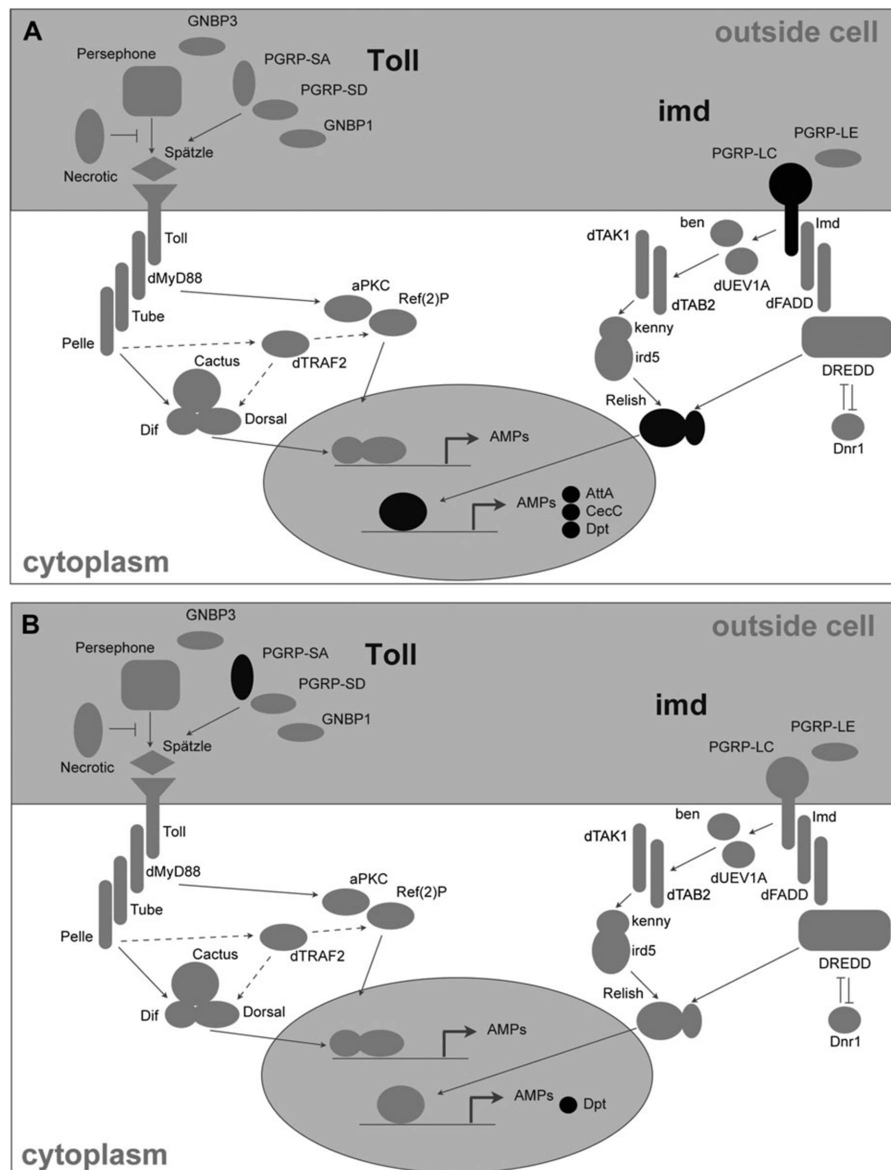

Fig. 5. Genes uniquely induced in F1 hybrids throughout humoral immune pathways. Genes in black are induced only in hybrids 6 (*A*) or 12 h (*B*) after infection with *Serratia marcescens*. Pathway genes and interactions included based on information in previous studies (Wassarman et al. 1995; Stronach and Perrimon 2002; Foley and O'Farrell 2004; Leclerc and Reichhart 2004; Arbouzova and Zeidler 2006; Ferrandon et al. 2007).

Using data from previous assays of genome-wide regulatory differences in interspecific *Drosophila* hybrids, Artieri et al. (2007) found significant correlations between the degree of dysregulation in the hybrids and the amount of sequence divergence between the parental species. We found no association between levels of amino acid divergence between *D. melanogaster* and *D. simulans* and levels of dysregulation in the hybrid immune gene expression as assessed by BeadChip analysis. We examined a restricted number of genes relative to Artieri et al. (2007), so the reduced statistical power of our test may have accounted for the difference. We did, however, find a correlation between the difference in parental expression levels and degree of hybrid dysregulation for the genes examined (ANOVA, $P = 0.0218$). This may indicate that for the groups of genes examined here, the functional context and regulatory control of a gene may better predict hybrid dysregulation of the immune response than will interspecific divergence at the sequence level.

Beyond examining correlations of individual gene properties, such as sequence divergence, with that gene's expression in hybrid flies, we find that genes belonging to different functional groups within the innate immune response show distinct patterns of dysregulation in hybrids. These functional groups, bearing genes whose products are involved in recognition, signaling, and effector roles in the response to infection, have been previously shown to have distinct patterns of sequence diversity—in terms of both naturally occurring variation within populations as well as divergence among species (Lazzaro et al. 2004, 2006; Sackton et al. 2007). The hybrid dysregulation that we see here indicates that interspecific divergence in immune

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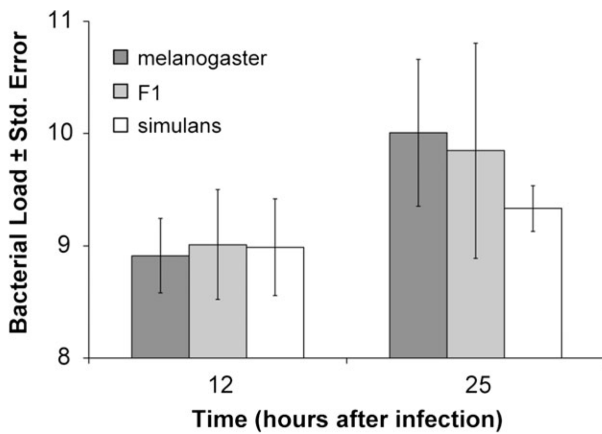

FIG. 6. Bacterial load after infection in *Drosophila melanogaster*, *D. simulans*, and F1 hybrid flies. Load represented as ln(colony-forming units per fly) at 12 and 25 h after infection with *Serratia marcescens*. Error bars shown represent the standard error of the mean.

pathway genes manifests differently among groups of immune genes not only in sequence differences but also in distinct regulatory patterns. In this case, the effector genes appear to be the most dysregulated in hybrids after infection. These tend to be overexpressed in the hybrids at all time points, and they are the group of genes most likely to show aberrant levels of induction or repression in the hybrids after infection. The patterns of disrupted expression in these downstream components of the immune response could indicate that regulatory differences in upstream components propagate and compound throughout the network, leading to the highest levels of dysregulation in these effectors.

The AMP effector genes often display unique patterns of expression in the hybrids, and they also show greater levels of hybrid overexpression than other functional groups. This directionality of the disruption may indicate that the regulatory breakdown in these hybrids is not random. We might expect some level of overexpression of immune genes in the hybrid flies relative to parents, because hybrids between *D. melanogaster* and *D. simulans* have been shown to have enlarged fat bodies (Dickinson et al. 1984), somewhat akin to the problems of assessing testis-specific expression in hybrids (Catron and Noor 2008). However, enlargement of the fat body in hybrids would not necessarily lead to distinct differences in hybrid dysregulation among the genes corresponding to separate functional groups within the immune pathways. The widespread overexpression of the downstream genes in the hybrid immune response may reflect some sort of basal regulatory mechanism of the pathways; perhaps the immune system in these flies is primed to have high AMP expression in response to infection.

Although an active response to infection may be beneficial in the face of regular exposure to microbes, it could be costly if it takes too much energy away from other vital tasks. To counter this, the *Drosophila* immune response includes strong negative feedback mechanisms. Because the hybrid flies in this study display a more active response to infection than the parental flies, this may represent a disruption of the negative feedback in the immune pathways, potentially due to divergent regulation of these mechanisms between the two parental species. If such disruption is present, we would expect for there to be decreased expression levels of genes with roles in repression of the immune response in hybrids. We do, in fact, find evidence for this—we observe that *Dnr1*, previously shown to repress activation of the imd pathway (Foley and O'Farrell 2004), is downregulated in hybrids more than in parents at both 6 and 12 h after infection, though most of these differences are not significant. It should also be noted that not all genes classified as members of the group of effectors are AMPs. Some of these genes encode proteins involved in stress response that can be transcribed in response to infection, yet may be induced as a result of other stresses—either at a systemic or at a cellular level. Although overexpression of these genes may reflect an overactive immune system, they could also reflect instances of dysregulation in the hybrids independent of the response to infection.

Although differences in the regulation of immune gene expression are evident between the parental species and with the F1 hybrids, it is also apparent that induction and repression patterns are at least somewhat consistent among genes that are closely positioned functionally in the humoral immune pathways. This does show that not only the expression changes after infection but also their relative magnitudes across genotypes are somewhat consistent with their positions in the humoral pathways, implying coordinated regulation of expression as well as evolution of that regulation. This is not entirely expected because the Toll and imd pathways are not transcriptional networks; upstream genes in the pathways do not directly control the transcription of their downstream neighbors. On the other hand, it is not surprising that there would be some level of coordination of expression of genes clustered within the pathways because the products of these genes interact. Furthermore, interacting proteins have been shown to be similarly expressed; Lemos et al. (2004) found the gene expression levels in pairs of proteins that interact to be significantly more correlated and significantly less polymorphic than random pairs of proteins in both *Saccharomyces cerevisiae* and *D. melanogaster*.

The observation that members of the Toll pathway are dysregulated in hybrids also raises questions about the effects of imprecise regulation of pleiotropic genes. With its role in the control of embryonic development as well as immune response in *Drosophila*, an intact Toll pathway is critical for proper function of multiple phenotypes at various life stages in the fly. Patterns of dysregulation of Toll genes could be associated with developmental differences in hybrids; distinct morphological and developmental features have in fact been observed in hybrids of these Drosophila species (Sturtevant 1920; Markow and Ricker 1991; David et al. 2002). Dysregulation of the Toll pathway in these interspecific hybrids has clearly extended beyond control of embryonic development, though; differences in induction and repression of Toll genes in hybrids as

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a result of bacterial infection in adult flies are indicative of regulatory divergence specific to the immune response.

As pairs of species diverge, the hybrids that they may form can bear dysfunctional phenotypes, ranging from inviability and sterility to more subtle differences in morphology or regulation. Through the juxtaposition of two diverged genomes, it is likely that numerous systems throughout a hybrid individual may be disrupted and that even seemingly subtle regulatory differences could have fitness consequences (Ortiz-Barrientos et al. 2007). Despite the extensive degree of dysregulation of gene expression, the most obvious indicator of disruption in F1 hybrids, namely, inviability of F1 males, appears to be associated with specific dysfunction of a few specific genes rather than being a consequence of genome-wide dysregulation (Barbash and Lorigan 2007). In this investigation of immune dysregulation of interspecific *Drosophila* hybrids, we have found that regulatory divergence of the innate immune system between *D. melanogaster* and *D. simulans* manifests distinctly in different portions of the immune response in F1 hybrids, with the most notable disparities appearing in the downstream pathway components. Furthermore, despite clear differences in patterns of expression and induction after infection in the F1 hybrids, these flies appear as immunocompetent as flies from the parental species, revealing a robustness of the immune function to even widespread regulatory perturbations and potentially highlighting an evolved ability of the immune networks to tolerate expression differences—at least in flies maintained in laboratory conditions.

Supplementary Material

Supplementary figures S1 and S2 and supplementary tables S1 and S2 are available at *Molecular Biology and Evolution* online (www.mbe.oxfordjournals.org).

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