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Article

Salmonella AvrA Coordinates Suppression of Host Immune and Apoptotic Defenses via JNK Pathway Blockade

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SUMMARY

Salmonellae **are bacterial pathogens that have evolved sophisticated strategies to evade host immune defenses. These strategies include the secretion of effector proteins into mammalian cells so as to subvert innate immune and apoptotic signaling pathways, thereby allowing *Salmonella* to avoid elimination. Here, we show that the secreted *Salmonella typhimurium* effector protein AvrA possesses acetyltransferase activity toward specific mitogen-activated protein kinase kinases (MAPKKs) and potently inhibits c-Jun N-terminal kinase (JNK) and NF-κB signaling pathways in both transgenic *Drosophila* and murine models. Furthermore, we show that AvrA dampens the proapoptotic innate immune response to *Salmonella* at the mouse intestinal mucosa. This activity is consistent with the natural history of *Salmonella* in mammalian hosts, where the bacteria elicit transient inflammation but do not destroy epithelial cells. Our findings suggest that targeting JNK signaling to dampen apoptosis may be a conserved strategy for intracellular pathogens.**

INTRODUCTION

All multicellular organisms possess the ability to respond to and manage microbial threats. This process involves transmembrane pattern recognition receptors that perceive the macromolecular signatures of microbes and set in motion cytoplasmic signaling cascades, which subsequently activate nuclear transcription factors and ultimately induce the transcriptional synthesis of antimicrobial, proinflammatory, and cytoprotective genes that serve to eradicate microbes and limit/repair damage. Alternatively, and often in parallel, microbial stresses induce apoptotic signaling, which in certain short lived cell types—such as intestinal epithelia—also serve to eradicate invasive pathogens. This framework of recognition, response, and repair, or focal apoptotic cell loss, has examples in plants, invertebrates, and mammals.

Key signaling pathways involved in these defenses against microbes include the mitogen-activated protein kinase (MAPK) pathways. The MAPKs function in a triple kinase sequence that involves the rapid and controlled relay of phosphorylation events to convey "alarm" signals (Roux and Blenis, 2004). Perception of diverse threats induces the upstream activators, kinases of the MAPKKK class. These proteins serve to activate the MAPKKs (or MEKKs), which subsequently activate the MAPKs and a downstream battery of immune and cell survival effector systems. MAPKKs such as MKK6 and MKK3 phosphorylate and activate members of the ERK and p38 MAPKs, respectively, which mediate primarily proliferative and cytoprotective responses. In contrast, the MAPKK MKK4/7 activates Jun N-terminal kinase (JNK), which is proinflammatory or, during prolonged activation, potently proapoptotic (Weston and Davis, 2007). In parallel, kinases of the IκB kinase family (IKKs)—which are closely structurally related to the MAPKKs—set in motion the NF-κB/Rel pathway by phosphorylation of IκB. This leads to proteasomal-mediated degradation of IκB and subsequent activation of NF-κB by nuclear translocation and induction of innate immune and antiapoptotic gene products. The involvement of MAPK family members in cellular responses to microbes are highly conserved across eukaryotic life, including animals and plants. Recognizable orthologs of the mammalian MAPKs, IKKs, and Rel factors are well described in vertebrates and arthropods (Hoffmann, 2003; Lemaitre and Hoffmann, 2007; Silverman and Maniatis, 2001).

Given the central role MAPKs and Rel pathways play in antimicrobial signaling, it is not surprising that some bacterial pathogens have evolved mechanisms to surmount their effects. Bacterial inhibition of NF-κB has been described at the level of IκB phosphorylation (Ruckdeschel et al., 1998), nuclear translocation (Kelly et al., 2004), and in past data from our laboratory at the level of IκB ubiquitination (Neish et al., 2000). Inhibition of MAPKs has been described in *Yersinia*, *Shigella*, and anthrax infections (Duesbery et al., 1998; Li et al., 2007; Orth et al., 1999). It is generally assumed that bacterial pathogens evolved these capacities to prevent or reduce the upregulation of the inflammatory response, thus blocking the influx of phagocytic leukocytes, and/or to activate or augment apoptotic pathways in immunoregulatory cells such as macrophages—both events likely permissive for bacterial proliferation and dissemination.

Bacteria can influence host cell signaling pathways via soluble effector proteins that are often translocated into the host cells or the environment via a "type III secretion apparatus" (Staskawicz et al., 2001). The effector proteins are generally assumed to usurp host cellular functions for the benefit of the invading organism and consequently are often designated as toxins or

"virulence factors." One family of secreted effector proteins with inhibitory effects on MAPKs is the YopJ/AvrA family. These proteins have been detected in a variety of bacteria that associate intimately with eukaryotic hosts. YopJ of *Yersinia enterocolitica* was shown to inhibit both multiple MAPK and NF-κB pathways, inducing rapid apoptotic death in infected macrophages (Orth et al., 1999; Ruckdeschel et al., 1998). Orthologs of this protein are found in other enteric pathogens such as *Vibrio parahemalyticus*, VopA (Trosky et al., 2004) and *Aeromonas salmonicida*, AopP (Fehr et al., 2006), and in a spectrum of plant pathogens (Ellis et al., 2007). Intriguingly, an ortholog is present in Rhizobia sp, a nitrogen-fixing plant symbiont (Freiberg et al., 1997), suggesting that members of this class of proteins are found in a wide variety of bacteria that mediate both pathogenic and symbiotic relationships, presumably by manipulating host innate immune signaling pathways.

Another member of this family is AvrA, a *Salmonella typhimurium* protein that is translocated into intestinal epithelial cells during the initial stages of invasion (Hardt and Galan, 1997). We have shown that AvrA overexpressed in transfected cells blocked NF-κB translocation and transcriptional activation of inflammatory effector genes (Collier-Hyams et al., 2002). To further understand its effects on cellular signaling pathways in vivo, we have exploited directed expression of AvrA in *Drosophila melanogaster*. Unlike experiments previously carried out with cultured mammalian cells, *Drosophila* allows investigation of the physiological effects of AvrA in an intact animal through well-defined genetic approaches using tissue-specific and inducible expression systems. We show that AvrA is a potent inhibitor of the *Drosophila* IMD pathway in response to Gram-negative infection, but only marginally affects the Toll pathway, which is activated in response to Gram-positive or fungal infection. Remarkably, immune blockade occurs without induction of apoptotic cell death characteristically seen during inhibition of host stress signaling pathways. We show that AvrA expression resulted in a potent blockade of JNK activation at the level of the MAPKK MEKK4/7 in both flies and human cells and inhibited JNK-mediated apoptosis. Consistent with this finding, we show that AvrA$^-$ *Salmonella* induced higher levels of inflammation, JNK activation, and epithelial apoptosis in infected murine intestine. Previously, experiments using extended colonization of cultured HeLa cells by nonproinflammatory *Salmonella* strains suggested that AvrA played a proapoptotic role (Collier-Hyams et al., 2002). However, here, using a pathogenic *Salmonella* strain and two whole-organism models, we provide data suggesting that the AvrA effector can inhibit innate immune responses in a eukaryotic host without the induction of apoptosis. Thus, an intracellular pathogen can "quiet" both inflammatory and apoptotic defenses.

RESULTS

AvrA Expression in the *Drosophila* Fat Body Suppresses Imd Pathway Activation

To investigate AvrA function in the context of a whole animal, we generated transgenic *Drosophila* harboring either wild-type *Salmonella typhimurium* protein AvrA or a catalytically inactive mutant form of AvrA (mAvrA) (C186A transition) under the transcriptional control of the UAS promoter, allowing tissue specific expression by crossing to GAL4 driver lines (Brand, 1994). Appropriate expression was determined by immunoblot analysis of fly tissue (Figure S1A available online). No toxicity was observed with AvrA expression in larval or adult animals. We examined whether expression of AvrA in the *Drosophila* fat body (the site of fly systemic immune response) could suppress innate immunity. Flies expressing AvrA had a greater mortality rate compared to flies expressing mAvrA following parenteral Gram-negative infections with *Escherichia coli* or *Erwinia carotovora* (Figure 1A), while control and mAvrA-expressing flies had similar mortality rates. Flies expressing AvrA infected with either the Gram-positive bacteria *Micrococcus luteus* or *Bauvaria basiana* fungal spores did not result in a detectable reduction in survival rates (Figure 1A), suggesting specificity of immunosuppression toward Gram-negative bacteria.

Fat body expression of AvrA inhibited the *Erwinia carotovora* stimulated upregulation of transcripts encoding the anti-Gram negative specific peptide Diptericin (Figure 1B), and also inhibited a Diptericin promoter driven reporter construct (Figure 1C) relative to the increased expression of Diptericin measured in control or mAvrA expressing flies. AvrA expression had a minimal inhibitory effect on the *Micrococcus luteus* stimulated upregulation of the anti-Gram positive-peptide Drosomycin (Figure 1B) or a Drosomycin reporter construct (Figure 1C).

To directly implicate AvrA activity on *Drosophila* immune signaling, we evaluated the activation of the Toll pathway transcription factor Dif and the *imd* transcription factor Relish in response to immune challenge. Upon activation, Dif and Relish are translocated to the nucleus where they initiate the upregulation of antimicrobial peptides production (diagrammed in Figure S2) (Lemaitre and Hoffmann, 2007). Larvae expressing either AvrA or mAvrA in their fat bodies were parenterally infected with either Gram-negative or Gram-positive bacteria. Relish translocation was completely inhibited in AvrA-expressing tissue following Gram-negative infection (Figure 1D), while AvrA had no inhibitory effect on Dif translocation (Figure 1E). Collectively, these data show that AvrA expression in the fat body of adult *Drosophila* selectively interferes with responses to Gram-negative infection (IMD pathway).

AvrA Expression Is Not Proapoptotic and Inhibits JNK/Bsk Phosphorylation and Caspase-3/Drice Activation in Response to Constitutive dTAK or Eiger Expression

The IMD/Relish pathway is tightly intertwined with apoptotic signaling intermediates such as the c-Jun N-terminal Kinase (JNK) (Liu and Lin, 2007). Furthermore, inhibition of Rel pathways is known to potently stimulate proapoptotic pathways in many systems and cell types. *Drosophila* offers an exquisitely sensitive system for assay of direct proapoptotic potential of expressed proteins by ectopic expression to the developing retina. We thus used the GMR-Gal4 driver to express AvrA immediately posterior to the morphogenetic furrow, allowing a spatial and temporal evaluation of apoptotic effects on the epithelial cells of the developing retina (Figure S3A). Surprisingly, AvrA (and the mutant form) had no discernable effects on gross appearance of the adult eye or any evidence of cell death (Figure 2A). This is in contrast to the "rough eye" phenotype induced as a result of the expression of two paralogs of AvrA: AopP from

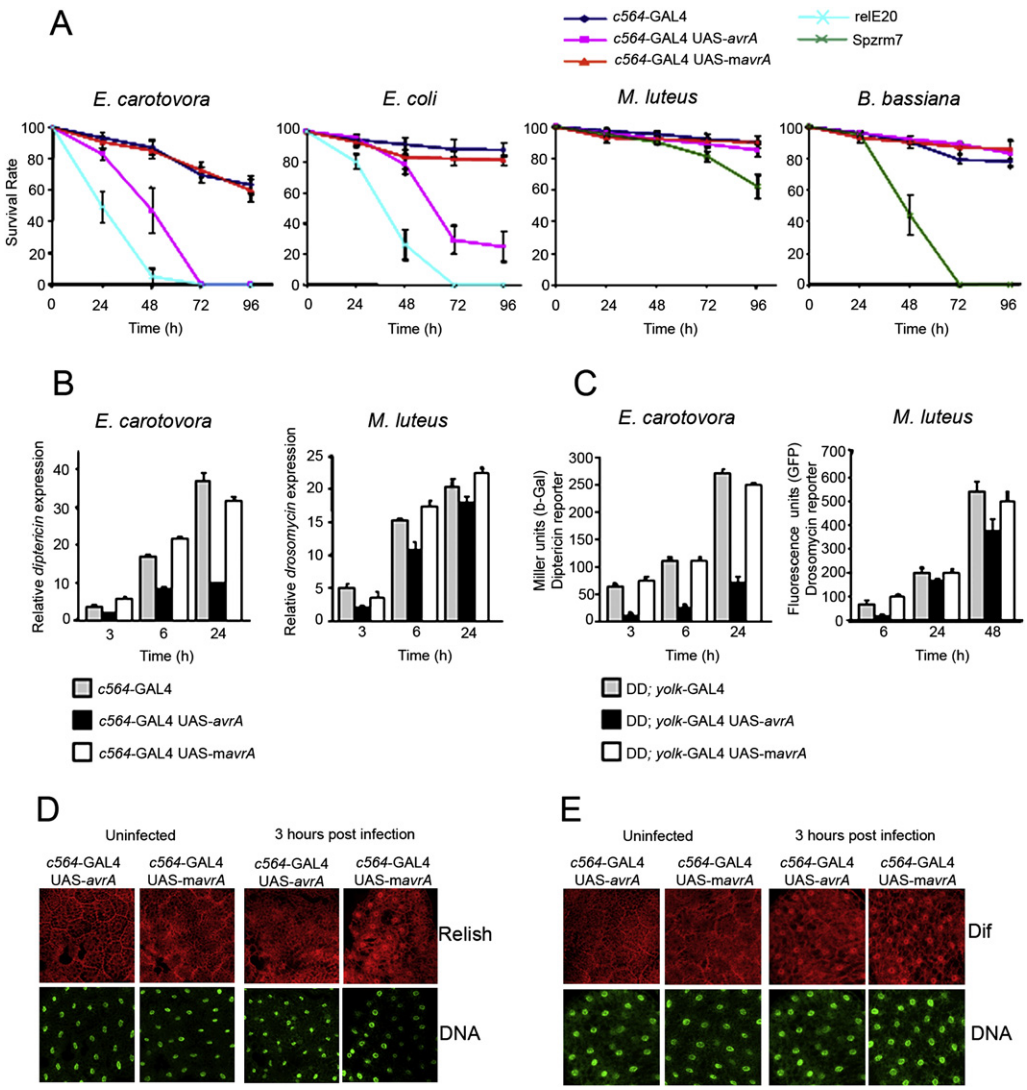

Figure 1. AvrA Expression in the *Drosophila* Fat Body Suppresses Innate Immunity in Response to Gram-Negative, but Not Gram-Positive, Infection

(A) Survival rates of the flies expressing AvrA (*c564*-GAL4 UAS-*avrA* or of the isogenic fly harboring UAS-m*avrA*), following parenteral Gram-negative *E. carotovora* or *E. coli* infection, and Gram-positive infection *Micrococcus luteus* or natural *Bauvaria bassiana* fungal infection. Relish mutant flies (rel^{E20}) susceptible to Gram-negative and *spatzle* (*spz*) null flies susceptible to fungal infections were used as controls. Results represent the average of triplicate studies and error bars represent standard deviation.

(B) Quantitative RT-PCR analysis of *diptericin* and *drosomycin* gene expression in flies infected with *E. carotovora* or *M. luteus*. PCR reactions were done in triplicate using two separate RNA preparations for each data point. Error bars represent SEM.

(C) β-galactosidase assays and GFP measurements of *E. carotovora*- and *M. luteus*-infected flies harboring DD1 (*y*, *w*, *P(ry+, Dpt-lacZ)*, *P(w+, Drs-GFP)*), *yolk*-GAL4;UAS-*avrA*, or of the isogenic fly harboring UAS-m*avrA*. Values for β-galactosidase activity and GFP determinations were normalized with respect to protein concentration. Assays were done in triplicate using two separate extract preparations for each data point. Error bars represent SEM.

(D) Fat body tissue from *E. caratavora* infected larvae was analyzed for Relish distribution by immunostaining using an antibody against the N-terminal domain of Relish.

(E) Fat body tissue from *M. luteus* infected third-instar larvae expressing AvrA or mAvrA was fixed and analyzed for Dif protein distribution by immunostaining using an anti-Dif antibody.

Aeromonas (Figure S3B; Figure 2A), which has been reported to exhibit inhibitory effects on NF-κB signaling but having no effect on MAPK signaling (Fehr et al., 2006), and the *Vibrio* protein VopA (Figure S3C; Figure 2B), reported to block ERK and JNK MAPK signaling while having no effect on NF-κB signaling (Trosky et al., 2007; Trosky et al., 2004). Importantly, the rough eye phenotype exhibited by AopP could be reversed by overexpression of *Drosophila* inhibitor of apoptosis (dIAP) and the IAP-like molecule p35, confirming this protein is directly proapoptotic (Figure 2A). Additionally, the VopA-mediated rough eye was reversed upon coexpression of activated Rolled (*Drosophila* ERK), confirming the ERK inhibitory role of VopA and showing that AvrA does not have inhibitory affects on the ERK MAPK pathway (Figure 2B). We also found that AvrA had no

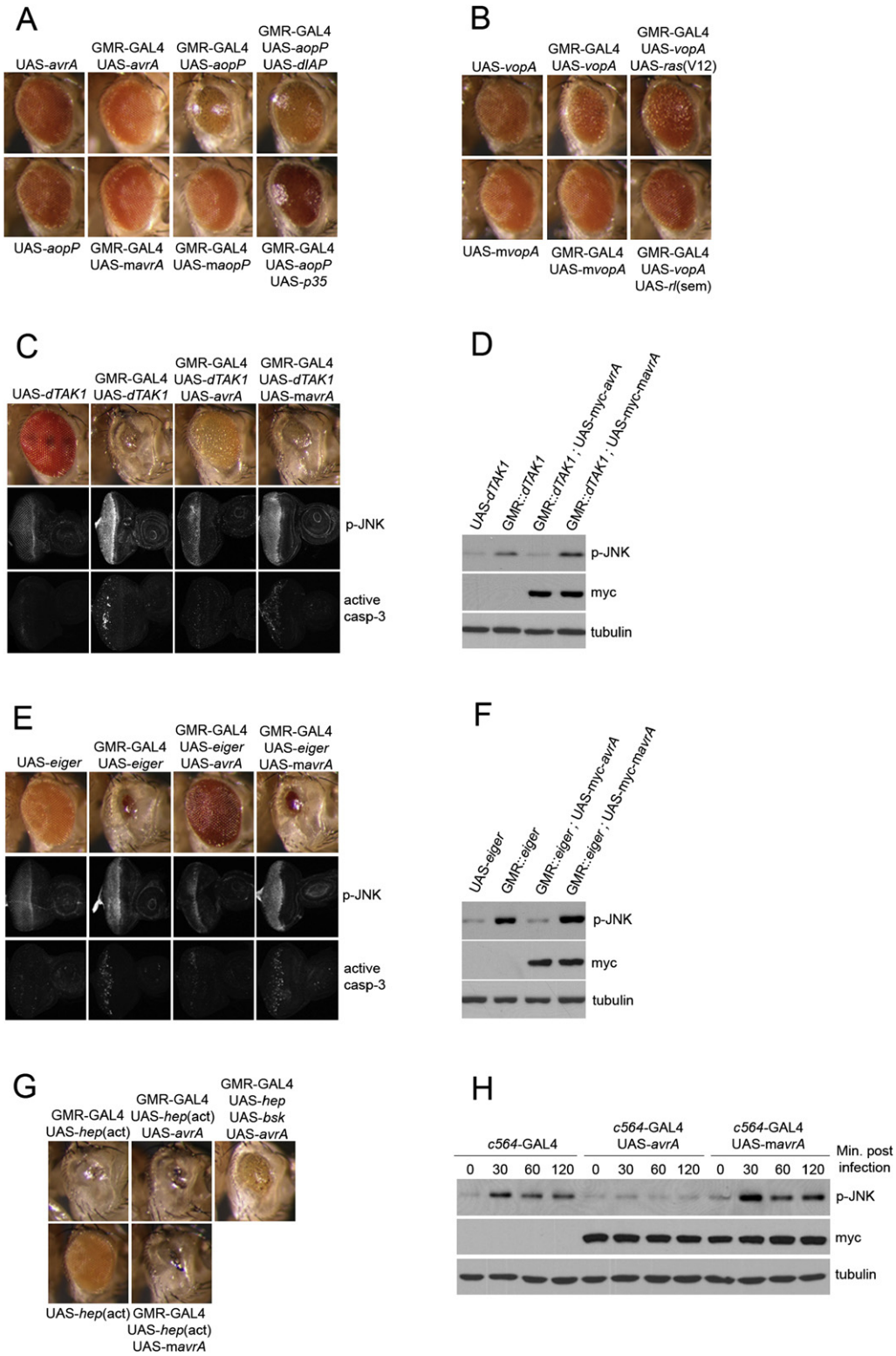

Figure 2. AvrA Expression Is Not Proapoptotic and Inhibits JNK/Bsk Phosphorylation and Caspase-3/Drice Activation in Response to Constitutive dTAK or Eiger Expression

(A) Phenotypes of Adult *Drosophila* Expressing AvrA, mAvrA, AopP, or mAopP under the eye-specific driver GMR-GAL4. Suppression of AopP-mediated small eye phenotypes by p35 or dIAP confirm the phenotype is a result of apoptotic events.

(B) Phenotypes of adult *Drosophila* expressing VopA or mVopA under the eye-specific driver GMR-GAL4. Suppression of VopA-mediated small eye phenotypes by overexpression of activated Rolled (*Drosophila* ERK) confirms the VopA rough eye phenotype is due to ERK pathway inhibition.

direct apoptotic effect when transfected in mammalian cell culture, even when cells were treated with TNF-α, whereas, consistent with previous reports, cells transfected with YopJ exhibited an increased number of cells with an apoptotic phenotype (Figure S4A), TUNEL (Figures S4B and S4C), and annexin V positivity (Figure S4D). Thus, we conclude that AvrA does not possess any direct proapoptotic activity, which is in striking contrast to paralogs encoded by other pathogens.

In flies and mammals, a key regulator of both inflammatory and apoptotic signaling is the MAPKKK TAK-1 (diagrammed in Figure S2) (Sato et al., 2005; Vidal et al., 2001). In flies, TAK-1 is a central regulator of the IMD immune pathway, and constitutive expression of TAK-1 signaling is proapoptotic (Takatsu et al., 2000). Immune-mediated (and TAK-1-dependent) apoptosis in flies and mammals can be mediated by prolonged and unopposed JNK signaling, events that can be physiologically antagonized by phosphatases induced via simultaneous and parallel Rel pathway activation, to allow immune responses in the absence of apoptosis (Kamata et al., 2005; Park et al., 2004). Third-instar retinal imaginal disk constitutive expression of dTAK-1 resulted in a strong small eye phenotype consistent with past reports (Leulier et al., 2002) (Figure 2C). Strikingly, AvrA coexpressed with dTAK-1 nearly completely suppressed the small eye phenotype, while mAvrA had no effect (Figure 2C). *Drosophila* Eiger/Wengen, the ortholog of mammalian TNF/TNF-R, is a potent and specific activator of JNK signaling and apoptosis (Moreno et al., 2002). Constitutive expression of Eiger in the retina resulted in an extreme small eye phenotype, consistent with past reports (Igaki et al., 2002) (Figure 2E). Constitutive expression of Eiger, an extra cellular cytokine, has the advantage of activating signaling without potential artifacts caused by intracellular constitutive expression of a cytoplasmic kinase. Again, AvrA expression was able to totally suppress this phenotype, while the mutant form did not. TAK-1 is a MAPKKK that functions as a JNK-KK. Hemipterous (MKK4/7 in mammals) is the subsequent MAPKK (JNK-K) in the JNK pathway (diagrammed in Figure S2). Constitutive expression of Hemipterous (UAS-hep) or Basket (UAS-bsk) in the *Drosophila* eye did not activate the JNK pathway and did not result in a small eye phenotype. However, concurrent constitutive expression of UAS-hep and UAS-bsk under GMR-GAL4 strongly activates the JNK pathway and is lethal in *Drosophila* at the pupal stage of development. AvrA expression in the GMR-GAL4 UAS-hep UAS-bsk genetic background resulted in a viable adult *Drosophila* exhibiting a mild rough eye phenotype (Figure 2G), whereas expression of mAvrA could not rescue the lethal phenotype. Finally, a constitutively active allele of Hemipterous (UAS-hep(act)) (Amino acid replacement: S326D and T330D in the kinase activation loop) also mediated a rough eye phenotype when driven by GMR-GAL4. Importantly, this phenotype could not be reversed by AvrA expression (Figure 2G). Together, these epistatic data indicate that AvrA inhibits the JNK pathway at the level of Hemipterous (MKK4/7) in *Drosophila*.

To confirm that apoptotic inhibitory effects were due to JNK pathway blockade, we subjected third-instar eye imaginal disks to immunostaining with anti-phospho-JNK antibodies. As expected, both eye disk-specific TAK-1 and Eiger-constitutive expression resulted in a strong activation of phospho-JNK immediately distal to the morphogenetic furrow that was concurrent with appearance of active capase-3 (Figures 2C–2F). Both phospho-JNK and active caspase-3 activity were suppressed by AvrA expression, but not mutant AvrA. Finally, levels of phosphorylated JNK in flies expressing AvrA in the fat body following Gram-negative infection were analyzed by immunoblot of fat body lysates. There was only a trace increase in phosphorylated JNK 30 min postinfection in flies expressing AvrA in the fat body compared to wild-type flies or those expressing mAvrA (Figure 2H), indicating AvrA could suppress microbially stimulated JNK signaling.

AvrA Expression Represses the JNK Pathway in Mammalian Cell Culture

To determine if our findings showing inhibition of JNK signaling extended to a mammalian system, we expressed AvrA and mAvrA in human 293T cells. Transfection efficiency was estimated at >80% by transfecting a plasmid harboring GFP coding sequence. Cells transfected with plasmids harboring AvrA or mAvrA as well as YopJ (which served as positive control) were stimulated with TNF-α, and cell lysates were analyzed by immunoblot for various endogenous signaling intermediates. While JNK was phosphorylated within 15 min of TNF-α addition in mAvrA and vector-only control, a complete blockade of JNK phosphorylation occurred in cells transfected with AvrA (Figure 3A). Note that unphosphorylated JNK increased in cells transfected with AvrA, but not mutant AvrA. In contrast, AvrA had no effect on TNF-α-induced phosphorylation of ERK or p38. Inhibition of TNF-α-induced phosphorylation of ERK, JNK, and, to a lesser extent, p38 occurred in cells transfected with YopJ (Figure 3A), whereas cells transfected with mYopJ had similar profiles to mAvrA-transfected cells (data not shown). Additionally, AvrA had no affect on TAK-1 phosphorylation in response to TNF-α stimulation (Figure 3B), indicating that AvrA inhibits the JNK pathway in mammalian cells distal to TAK-1. Also, AvrA expression resulted in partial stabilization of IκB-α (Figure 3C) while having little or no effect on TNF-α induced IκB-α phosphorylation consistent with past experiments (Collier-Hyams et al., 2002). Finally, AvrA, but not mAvrA expression, could repress JNK-dependent reporter gene expression while

(C) Phenotypes of adult *Drosophila* eyes resulting from dTAK overexpression and from a combination of dTAK1 and AvrA overexpression, with genotypes indicated. Third-instar larval eye disks were immunostained for p-JNK and active caspase-3.
(D) Immunoblot analysis for p-JNK levels in larval eye imaginal disks from genotypes examined in (C).
(E) Phenotypes of adult eyes resulting from the Eiger overexpression and from a combination of Eiger and AvrA overexpression with genotypes indicated. Third-instar larval eye disks were immunostained as above.
(F) Immunoblot analysis for p-JNK levels in larval eye imaginal disks from genotypes examined in (E).
(G) Phenotypes of adult *Drosophila* eyes resulting from activated hep overexpression and from a combination of activated hep and AvrA overexpression in the *Drosophila* eye.
(H) Parenteral *E. caratavora* infection of third-instar larvae expressing AvrA in the fat body. Larval fat body was dissected at time points up to 1 hr postinfection and analyzed for p-JNK by immunoblot.

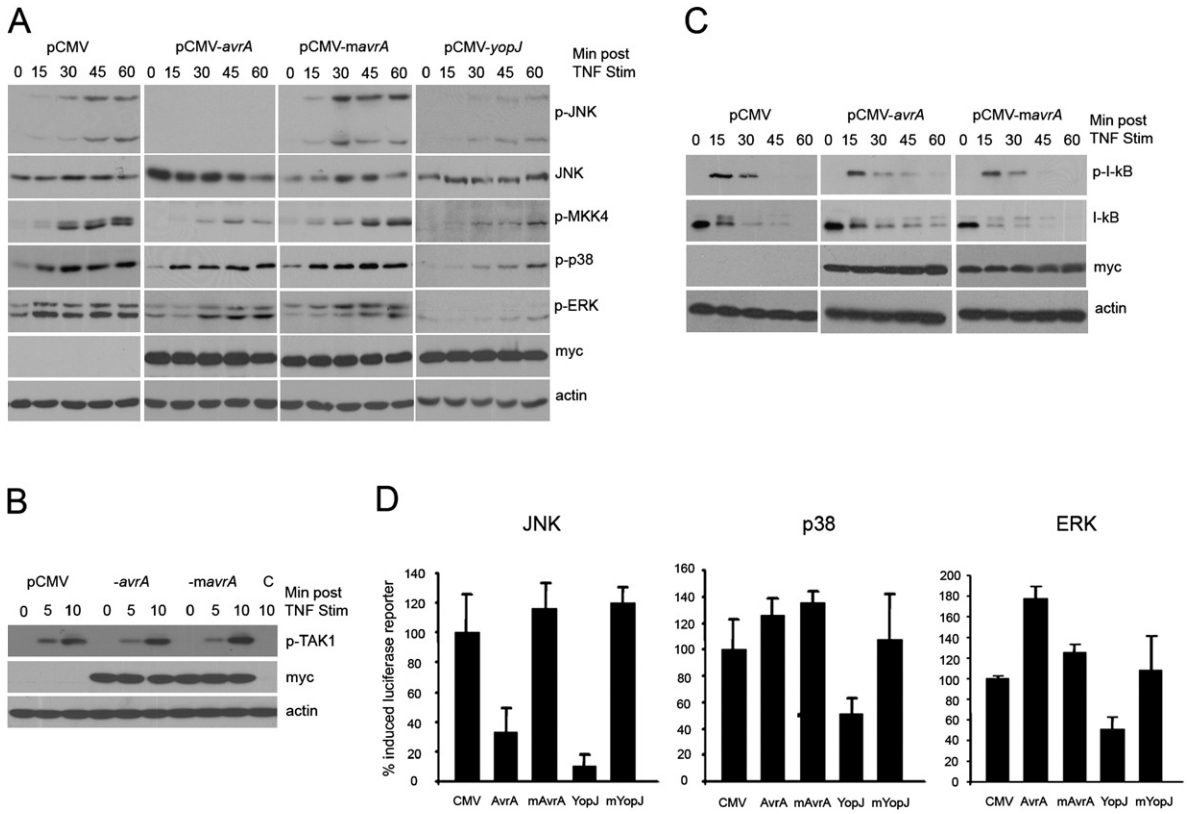

Figure 3. AvrA Expression Represses the JNK Pathway in Mammalian Cell Culture

(A) 293T cultures cells transfected with vector control or plasmids harboring AvrA, mAvrA, or YopJ were stimulated with 10 ng/ml TNF-α over 1 hr and assayed by immunoblot with the indicated antibodies.

(B) 293T cultured cells transfected with vector control or plasmids harboring AvrA or mAvrA were stimulated with 10 ng/ml TNF-α over 10 min in the presence of 10 ng/ml Calyculin A and assayed by immunoblot using anti-phospho TAK1 antibody.

(C) 293T cultured cells transfected with vector control or plasmids harboring AvrA or mAvrA were stimulated with 10 ng/ml TNF-α over 1 hr and assayed for IκB or phospho IκB.

(D) JNK, p38, or ERK signaling pathways activation levels in 293T cells were measured by PathDetect (Stratagene) luciferase reporter gene assay in the presence of plasmids harboring AvrA, mAvrA, YopJ, or mYopJ, respectively. Luciferase reporter gene assays were done in triplicate using two separate extract preparations for each data point. Experimental results are recorded as percent expression relative to measurements in cells expressing vector alone. Error bars indicate SEM.

having no measurable affect on p38 or ERK pathway signaling. In comparison, YopJ exhibited the expected inhibition of the JNK, p38, and ERK pathways (Figure 3D). Thus, AvrA is a potent inhibitor of the JNK pathway with partial effects on the NF-κB pathway.

AvrA Inhibits JNK Pathway Activation at the Level of MKK4/7 by Acetylation

In *Drosophila*, AvrA rescued dTAK1-mediated rough eye phenotype, but not the small eye phenotype caused by overexpression of an activated form of Hemipterous (*Drosophila* MKK4/7). Additionally, AvrA did not inhibit TAK1 phosphorylation. This suggests AvrA inhibits the JNK pathway at the level of the MAPKK. Thus, we probed TNFα-stimulated cell lysates with an antibody against phospho-MKK4 and found that activation of this JNK kinase was significantly reduced (Figure 3A). Furthermore, we activated the JNK pathway in human-cultured cells with plasmids harboring JNK1 and MKK4 or MKK7. Previous reports have shown cotransfection of JNK1 and MKK7 induced JNK phosphorylation (Lei et al., 2002). AvrA cotransfection, but not mAvrA, could totally abolish both MKK4- or MKK7-mediated JNK phosphorylation (Figures 4A and 4B), thus indicating that AvrA mediates JNK pathway inhibition at the level of MKK4 and MKK7.

Previous reports have shown that the AvrA homolog YopJ mediates inhibition of the MAPK pathway by acting as an acetyltransferase, using acetyl-coenzyme A (CoA) to modify both Ser and Thr residues in the activation loop of MKK6 and IKKβ, thereby blocking phosphorylation (Mittal et al., 2006; Mukherjee et al., 2006). In order to determine whether AvrA has similar acetyltransferase activity on MKK4, we analyzed tryptic digests from lysates obtained by the co-transfection of pCMV-mycMKK4 with pcDNA-flagAvrA by mass spectrometry. Data revealed an increase of 42 amu at K^{260} and Thr^{261} active site residue of the MKK4 consistent with an O-acetylation of an amino acid residue (Figure 4C and Figure 4D). We found no evidence of acetylation at the Ser^{257} active site residue in tryptic digests including this residue. Control experiments where MKK4 was transfected

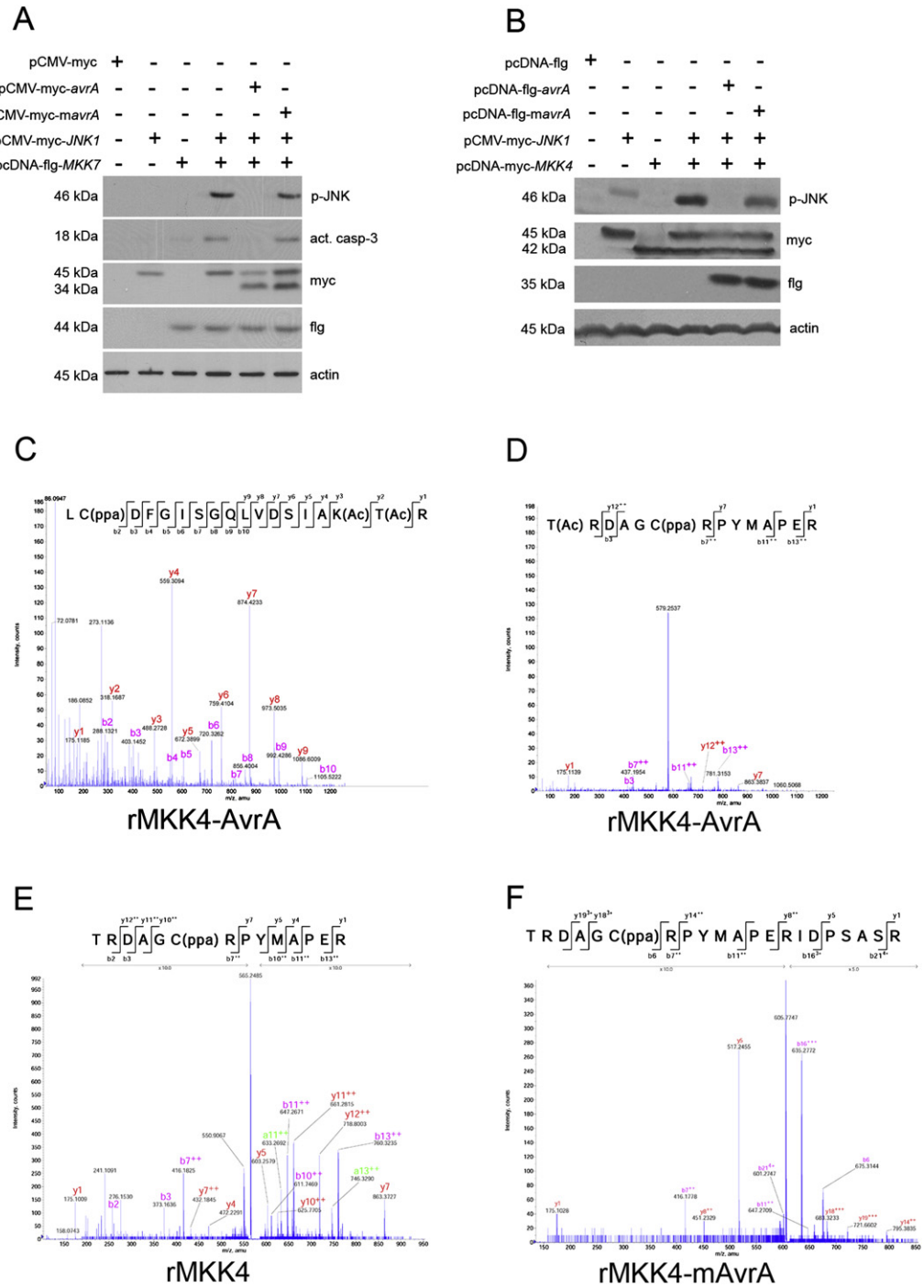

Figure 4. AvrA Inhibits JNK Pathway Activation at the Level of MKK4/7 by Acetylation

(A) Immunoblot analysis for phospho-JNK activity in lystaes of HeLa cultured cells transfected with the indicated plasmids. Myc antibody detects JNK at 45 kDa and AvrA/mAvrA at 34 kDa.

(B) Immunoblot analysis for phospho-JNK activity in lystaes of HeLa cultured cells transfected with the indicated plasmids. Myc antibody detects JNK at 45 kDa and MKK4 42 kDa. Flag antibody detects AvrA/mAvrA at 35 kDa.

(C–F), Electrospray ionization (ESI) tandem mass spectrometry (MS/MS) spectra of modified tryptic peptide (C) (mass-to-charge ratio [m/z] of 693.37 [z = 3]) and peptide (D) (m/z of 579.27 [z = 3]) from MKK4-AvrA, peptide (E) (mass-to-charge ratio [m/z] of 565.27 [z = 3]) from rMKK4, and peptide (F) (mass-to-charge ratio [m/z] of 605.79 [z = 4]) from MKK4-mAvrA. The b and y ions are marked on the MS/MS spectra. The amino acid sequence for each peptide is shown. Putatively acetylated residues are designated by Ac in the sequence. The abbreviation ppa (proprionamide) is for alkylation of the cysteine residues by acrylamide from the gel.

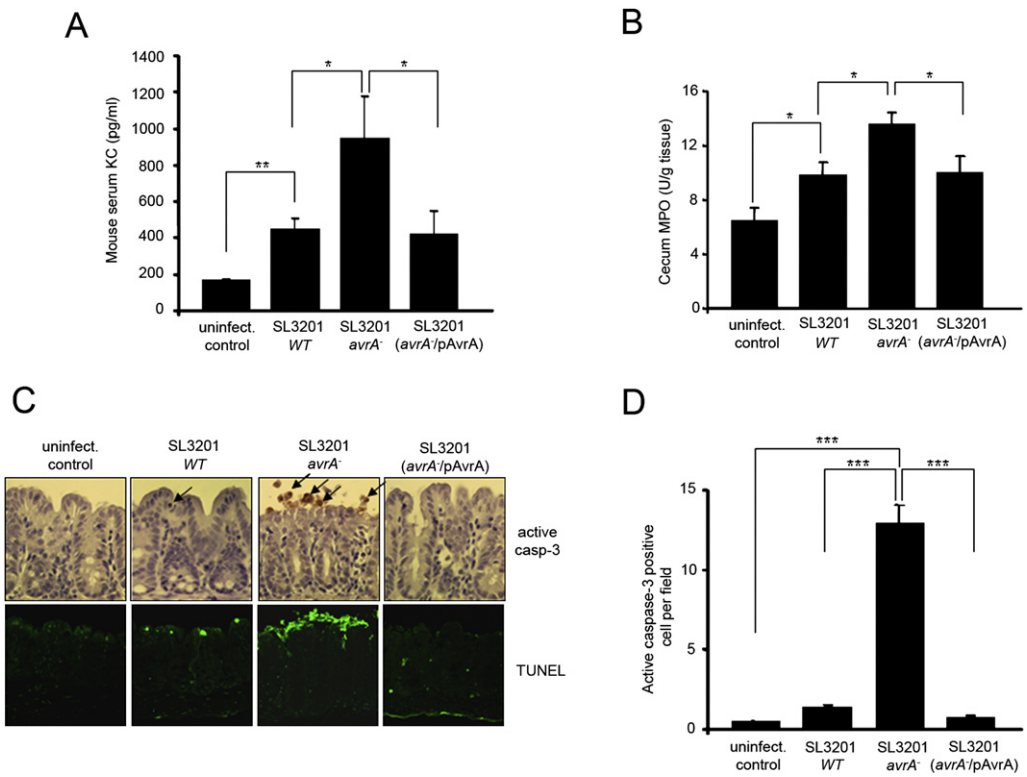

Figure 5. *Salmonella* AvrA Mediates Suppression of Epithelial Innate Immunity and Apoptosis during Natural Infection

Streptomycin-pretreated mice were inoculated with 10^8 CFU WT *Salmonella* SL3201 or isogenic AvrA$^-$ mutant, or a AvrA$^-$ mutant complemented with wild-type *avrA*$^+$ (AvrA$^-$/pAvrA) for six hours. (A) KC ELISA from peripheral serum and (B) Myeloperoxidase (MPO) assay for colonic mucosal lysates. (C) Immunohistochemical staining for active caspase-3 and TUNEL stain in colonic mucosa 6 hr post-oral infection with the indicated strain. Five mice were analyzed in each experimental group. (D) Quantification of active-caspase-3 positive cells from (C). *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$.

with vector alone or catalytically inactive mAvrA showed no active site residue modification (Figure 4E and Figure 4F). Thus, AvrA can act as an acetyltransferase in the manner described for other members of this family (Mittal et al., 2006; Mukherjee et al., 2006). Also note that the constitutively active form of *Drosophila* Hemipterous (MKK4/7) could not be suppressed by AvrA (Figure 2G), presumably because the T-to-D transition could not be O-acetylated.

Salmonella AvrA Mediates Suppression of Epithelial Innate Immunity and Apoptosis during Natural Infection

To evaluate the effect of AvrA in natural infection in mammals, we turned to a recently developed model of intestinal Salmonellosis using streptomycin-pretreated mice. This model has been widely validated and has been used by ourselves and others as a system that faithfully replicates the acute inflammatory events on enteric Salmonellosis (Barthel et al., 2003; Vijay-Kumar et al., 2006). We used wild-type *Salmonella* SL3201, an AvrA$^-$ isogenic *Salmonella* mutant bearing a nonpolar insertional mutation in the chromosomal *avrA* gene, and the AvrA$^-$ mutant complemented with wild-type *avrA*$^+$ (AvrA$^-$/pAvrA). Murine infection with the AvrA$^-$ *Salmonella* resulted in elevated levels of serum KC (the murine ortholog of the neutrophil specific chemoattractant IL-8) (Figure 5A), relative to infection with isogenic wild-type *Salmonella* or the *avrA*$^+$ complemented mutant, again consistent with AvrA mediating a repression of innate immune signaling. Additionally, increased neutrophil influx into the intestinal mucosa was seen in mice infected with AvrA$^-$ *Salmonella* as measured by myeloperioxidase (MPO) (Figure 5B), a histochemical marker of neutrophils.

We next sought to determine effects of AvrA on epithelial apoptosis in the context of natural infection. Infection with wild-type *Salmonella* or the *avrA*$^+$-complemented AvrA$^-$ mutant resulted in scattered apical caspase-3 and TUNEL-positive cells, consistent with our previous observations (Vijay-Kumar et al., 2006) (Figures 5C and 5D). Strikingly, the isogenic AvrA$^-$ mutant elicited a marked increase in both caspase-3 and TUNEL-positive cells at this time point. From this data, we conclude that *Salmonella* AvrA serves to suppress both epithelial innate immunity and apoptosis during the initial phase of *Salmonella* interaction with the intestinal mucosa.

Finally, we evaluated the effects of AvrA on JNK pathway activation during initial events of infection using a murine mucosal ex vivo model. Intestinal segments were infected for 5 min with either wild-type *Salmonella*, AvrA$^-$ mutant *Salmonella*, or the AvrA$^-$ mutant complemented with wild-type *avrA*$^+$. Infection with the AvrA$^-$ *Salmonella* resulted in elevated phospho-JNK levels in cells at the apical regions of the intestinal villi, whereas infection with the other bacteria did not (Figure 6A). Additionally, immunoblot analysis of scrapings from intestinal mucosa showed increased levels of phospho-JNK following infection by AvrA$^-$ mutant bacteria, compared to the wild-type or the

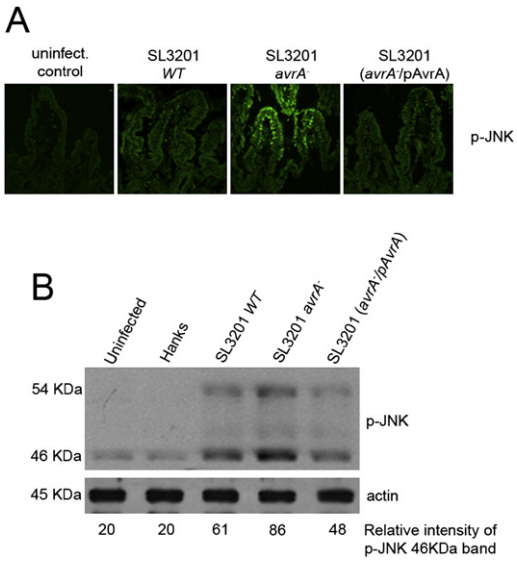

Figure 6. *Salmonella* AvrA Mediates Suppression of Phospho-JNK Activation in the Murine Intestine

Murine intestine was dissected and infected with 10^9 CFU of WT *Salmonella* SL3201, isogenic AvrA$^-$ mutant, or an AvrA$^-$ mutant complemented with wild-type *avrA*$^+$ (AvrA$^-$/pAvrA) for 5 min.

(A) Immunohistochemical staining for p-JNK in intestinal mucosa 5 min postinfection with the indicated strain.

(B) Immunoblot analysis of mouse intestinal epithelial cells infected for 5 min with the indicated *Salmonella* strain with a p-JNK antibody. Relative intensities of p-JNK band at 46 kDa were measured using Scion Image beta analysis program (Scion Corporation).

avrA$^+$-complemented AvrA$^-$ mutant (Figure 6B). Collectively, these data indicate AvrA suppresses phospho-JNK activation during the initial phase of *Salmonella* contact with intact epithelia.

DISCUSSION

Drosophila has been a workhorse of modern biology for nearly a century. The ease of working with this species and the "genetic tractability" developed over years has led to the use of *Drosophila* in many areas of experimental biology, including development, cell biology, neuro-, and behavioral biology. However, only recently has this organism been recognized to possess an antimicrobial defense system with striking functional and structural homology to mammalian innate immune networks, thus allowing this model system to be applied to problems in innate immunology (Lemaitre and Hoffmann, 2007). Recently, genetic approaches to the study individual prokaryotic or viral effector molecules have been attempted (Guichard et al., 2006; Jia et al., 2006; Leulier et al., 2003) and have shown utility in elucidating effects of noneukaryotic proteins on an intact animal.

In this report, we examined AvrA in a transgenic *Drosophila* genetic model. Our experiments revealed that AvrA mediated the selective inhibition of the IMD pathway in *Drosophila*. *Drosophila* expressing AvrA were highly susceptible to Gram-negative infection, did not upregulate the Gram-negative-specific peptide Diptericin, and failed to activate the Gram-negative-specific Relish factor (assayed by cytoplasmic to nuclear translocation) upon bacterial challenge. The selective inhibition of the IMD pathway, with its specificity toward Gram-negative bacteria, is consistent with the hypothesized function of a *Salmonella* effector protein, itself a Gram-negative pathogen. The IMD pathway, unlike the Toll pathway, requires activation of MAPKK signaling intermediates, including Ird5 (*Drosophila* IKK-β) (Silverman et al., 2000) and Kenny (*Drosophila* IKK-γ) (Rutschmann et al., 2000). IMD pathway inhibition by AvrA is highly consistent with the proposed activities of the AvrA/YopJ effectors as inhibitors of the MAPKK class of enzymes. Additionally, our data support previous reports where infection of *Drosophila* mbn-2-cultured cells with *Salmonella typhimurium* dampened AMP activation via inhibition of the Imd/Relish pathway (Lindmark et al., 2001).

The effects of the *Salmonella typhimurium* protein AvrA on cellular innate immune signaling has been studied using mammalian cell-based assays (Collier-Hyams et al., 2002). These experiments showed that overexpressed AvrA suppressed the canonical NF-κB pathway by inhibiting nuclear translocation of the p50/p65 Rel proteins, resulting in blockade of NF-κB-dependent promoter activation. These observations are concordant with the AvrA-mediated inhibition of Relish translocation and suppression of Rel-dependent diptercin activation in the fat body of *Drosophila*, illustrating the conservation of AvrA-mediated effects on this pathway. In both our past manuscripts (Collier-Hyams et al., 2002) and the current work, we did not see inhibition of IκB-α phosphorylation but did observe stabilization of IκB-α. This is similar to data obtained with overexpressed YopJ, which while mediating marked inhibition of IKK-β kinase activation and subsequent stabilization of the IκB-α protein, showed only minimal effects on endogenous IκB-α phosphorylation (Mittal et al., 2006), possibly suggesting that IKKs have functional targets in the NF-κB pathway distinct from the classic dual serine motif of IκB-α.

There are three orthologs of AvrA with described activities toward the MAPKK family and IKKs, and all serve to repress innate immune responses. YopJ of Yersinia has a wide range of inhibitory activities against ERK, p38, JNK, and IKK (Orth et al., 1999), VopA of *Vibrio parahemalyticus*, which has been shown to inhibit ERK, p38 and JNK, but not IKK (Trosky et al., 2007; Trosky et al., 2004), and AopP of *Aeromonas salmonicida*, which is apparently specific to IKK (Fehr et al., 2006). Our data is highly supportive of the concept that these proteins can differentially and variably inhibit members of the MAPKKs and IKKs. In biochemical and functional analysis directly comparing the ability of YopJ and AvrA to repress the spectrum of MAPK pathways in mammalian cells, we showed that AvrA was especially inhibitory to JNK, with lesser activity toward IKK, while YopJ repressed all three MAPK pathways and IKKs. These observations indicate that bacteria can impinge on innate immune pathways by targeting multiple different MAPKKs, highlighting the interrelated role these pathways have on immune signaling.

Our most unexpected and striking finding was the ability of AvrA, unique among this class of effectors, to suppress apoptosis. AvrA did not result in apoptosis in *Drosophila* fat body constitutively or during systemic infection (data not shown). AvrA did not induce apoptosis during constitutive or induced expression in *Drosophila* eye or wing discs, in striking contrast to the expression of AopP, which was directly and potently proapoptotic in these tissues and in transfection experiments in mammalian cell culture. We show YopJ to be potently

proapoptotic in cultured mammalian cells in the presence of TNF-α, again in contrast to AvrA, which had no proapoptotic activity even in the presence of this cytokine. We previously had reported that infection of cultured HeLa cells with AvrA mutants in the background of *Salmonella* strains/species that are nonproinflammatory in human cells (*Salmonella typhimurium* PhoP^c, and *S. pullorum*) resulted in an increase in the apoptotic cell population (Collier-Hyams et al., 2002). However, Ye et al. described reduced apoptosis in murine intestine infected with these strains (Ye et al., 2007). While these in vivo experiments did not use a proinflammatory pathogenic wild-type host strain, the data are consistent with AvrA mediating an antiapoptotic role and suggests results obtained in cell culture represent a situation idiosyncratic to in vitro systems. In the context of proinflammatory enteropathogenic Salmonellosis, our results suggest AvrA represses apoptosis during the epithelial step of the infectious process.

As mentioned, members of the YopJ-like family of proteins are well described as having differential inhibitory effects on substrates at the MAPKK level (Mittal et al., 2006; Mukherjee et al., 2006; Trosky et al., 2007). This selectivity of substrate specificity likely accounted for the striking differential effects on apoptosis. In general, JNK is thought of as a proapoptotic pathway, while ERK and p38 (and NF-κB) are understood as mediating survival signals (Weston and Davis, 2007). Our data is supportive of this concept, with AvrA as a JNK inhibitor acting as a survival effector protein. Furthermore, we show AvrA is able to repress apoptotic signaling from constitutively active Eiger and the JNK-KK TAK1, consistent with the known proapoptotic role of sustained JNK activation (Kamata et al., 2005).

Recent biochemical studies showed YopJ and VopA act as an acetyltransferase, using acetyl-coenzyme A (CoA) to modify critical serine and threonine residues of target MAPKKs, thereby blocking phosphorylation and activation of the protein (Mittal et al., 2006; Mukherjee et al., 2006; Trosky et al., 2007). The reported biochemical target of VopA coincides with functional repression exhibited in our genetic data showing VopA-mediated ERK pathway inhibition at the level of dSor (*Drosophila* MKK6). Our molecular data strongly suggested that AvrA inhibits JNK signaling at the level of the JNK-K (MKK4/7 in mammals, Hemipterous in *Drosophila*). Consistently, we used mass spectroscopy to evaluate recombinant MKK4 when coexpressed with AvrA, demonstrating the novel O-acetylation on the Thr residue of the MKK4 activation loop as expected for this class of bacterial proteins. That a modification of the activation loop is responsible for the apoptotic repressive action of AvrA is also strongly supported by genetic data showing a constitutively active JNK-K (Hemipterous), which is defined by a phosphomimetic mutation of the Thr in the activation loop, causes a rough eye that can not be suppressed by AvrA, while JNK-KK (TAK)-mediated rough eye is suppressed (Figure 2G). Other reports have concluded that the YopJ/AvrA proteins function as deubiquitinating enzymes (Sweet et al., 2007; Ye et al., 2007; Zhou et al., 2005). Such a biochemical activity may occur in parallel with acetyltranferase-mediated inhibition and is not inconsistent with our observed effects on signaling pathways.

The overall paradigm of defensive response or altruistic cell loss, as well as the associated biochemical signaling pathways, is highly conserved across metazoans, including mammals and invertebrates (Hoffmann, 2003; Silverman and Maniatis, 2001; Weinrauch and Zychlinsky, 1999). Collectively, our data show complete concordance of the ability of AvrA to influence specific MAPKK-mediated signaling, immune activation, and apoptosis in widely disparate eukaryotic in vivo systems. *Salmonella* induces a brisk, but nondestructive, acute inflammatory response in the epithelia, both in natural human and model murine infections. However, murine infection with the AvrA null mutant resulted in increased cellular inflammation and a massive increase in epithelial apoptosis. Thus, AvrA activity may account for the self-limited inflammation seen in Salmonellosis, in contrast to the more destructive epithelial effects characteristic of infection by other enteric pathogens such as *Shigella* and EHEC (Sansonetti and Di Santo, 2007). The AvrA protein apparently allows the invading bacterium to dampen innate immune signaling but also prevents the apoptotic elimination in cells that have perceived microbial compromise.

Conversely, the greater proapoptotic activity of the AvrA ortholog in *Yersinia*, YopJ (Mukherjee et al., 2006) (ascribed to its more promiscuous range of MAPKK inhibitory activity) is consistent with that organism's ability to rapidly kill phagocytic cells and mediate a more virulent infection. It appears *Salmonella* has refined a similar biochemical activity to mediate a more benign cellular consequence. Indeed, the existence of related biochemical effectors in plant symbionts (Staskawicz et al., 2001) illustrates the exquisite fine tuning of host responses that bacteria can engender.

EXPERIMENTAL PROCEDURES

Experimental Infection

Infections were performed by stabbing adult *Drosophila* or third-instar larvae with a thin tungsten needle dipped in a concentrated pellet of *Erwinia carotovora*, *Escherichia coli*, or *Micrococcus luteus*. Fungal spore infections were done using the natural fly pathogen *Bauvaria bassiana*. Cultures of *B. bassiana* were propagated in fly vials, whereupon anesthetized flies were passaged into the vial and covered in fungal spores by shaking the vial for 2 min. Survival rates were determined by counting survivors at time intervals. Larval infection was undertaken by parenteral infection with microbially infected needle. Infected larvae were immediately placed in a Petri dish containing water-saturated tissue paper to avoid desiccation.

TUNEL Assay

Apoptotic cells were detected by TUNEL assay using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH) according to manufacturer's instructions. Cultured HeLa cells were grown on 10 mm diameter cover slips in Petri dishes. Following plasmid transfection and treatment with TNFα, cover slips were removed from Petri dishes, inverted, and sequentially placed on 100 μl TUNEL assay reagents. Cover slips were then mounted on slides, and cells viewed by Confocal microscopy. For analysis of TUNEL positive cells in the intestine of infected mice, paraffin embedded caecal tissue sections were mounted on slides, and sequentially treated with TUNEL assay reagents. Tissue sections were then over layed with cover slips and viewed by Confocal microscopy.

Murine Infections

The creation of an *avrA* null of *Salmonella* SL3201 mutant is previously described (Collier-Hyams et al., 2002). Pathogen-free female BALB/cj mice (6–8 weeks) were procured from Jackson Laboratories (Bar Harbor, ME). Animals were deprived of food and water for 4 hr before treatment with 7.5 mg of streptomycin by oral gavage. Afterward, animals were supplied with food and water ad libitum. At 20 hr poststreptomycin treatment, water and food were withdrawn again for 4 hr before the mice were infected with

10^8 CFU wild-type *Salmonella* SL3201, the isogenic *avrA*$^-$ mutant form of SL3201, or the mutant complemented with plasmid expressed AvrA. Construction of these mutants is as described in Collier-Hyams et al. (2002). At 6 hr postinfection, blood samples were collected, and serum KC levels were determined using a mouse CXC chemokine KC DuoSet kit according to manufacturer's instructions (R&D Systems, Minneapolis, MN). Thereafter, ceca were removed, and neutrophil infiltration into tissue was quantified by measuring MPO enzyme activity (a marker for neutrophils) by biochemical assay. Histological sections of ceca were prepared from five infected animals per treatment. Sections were assessed for active caspase-3 activity by numeration by the average number of positive cells in forty 200× fields per infected animal. Animal experiments were approved by the Emory University institutional ethical committee and performed according to the legal requirements.

For ex vivo analysis of phospho-JNK activation in murine gut, small intestines were removed from 8-week-old BALB/cj mice, and 2 cm segments were immediately placed in Hank's buffer at 37°C. The segments were then infected with *Salmonella* cultures (10^9 CFU/ml) for time periods up to 1 hr. Infected and control segments were opened along the mesenteric border, and epithelial tissue was scraped and lysed by homogenization in RIPA buffer (100 mg tissue per milliliter of buffer) and centrifuged at 16,000 rpm. for 20 min at 4°C. Protein concentrations of supernatants were determined by protein assay (Bio-Rad), and equal amounts of protein were diluted with denaturing SDS-PAGE loading buffer and assayed by immunoblot.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and four supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/3/4/233/DC1/.

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