CUTTING EDGE

Cutting Edge: *Salmonella* AvrA Effector Inhibits the Key Proinflammatory, Anti-Apoptotic NF-κB Pathway[1]

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Secreted prokaryotic effector proteins have evolved to modulate the cellular functions of specific eukaryotic hosts. Generally, these proteins are considered virulence factors that facilitate parasitism. However, in certain plant and insect eukaryotic/prokaryotic relationships, effector proteins are involved in the establishment of commensal or symbiotic interactions. In this study, we report that the AvrA protein from *Salmonella typhimurium*, a common enteropathogen of humans, is an effector molecule that inhibits activation of the key proinflammatory NF-κB transcription factor and augments apoptosis in human epithelial cells. This activity is similar but mechanistically distinct from that described for YopJ, an AvrA homolog expressed by the bacterial pathogen *Yersinia*. We suggest that AvrA may limit virulence in vertebrates in a manner analogous to avirulence factors in plants, and as such, is the first bacterial effector from a mammalian pathogen that has been ascribed such a function. ***The Journal of Immunology*****, 2002, 169: 2846–2850.**

Previously, we reported that colonization of model human epithelia in vitro with *Salmonella typhimurium* PhoP^c (1) or *Salmonella pullorum* strains, which are not pathogenic or proinflammatory in a variety of in vivo and in vitro assays, prevented activation of the proinflammatory/anti-apoptotic NF-κB pathway when stimulated by multiple proinflammatory agonists, including virulent *S. typhimurium* (2). These effects were mediated by a block in IκB-α ubiquitination while IκB-α phosphorylation was unaffected.

Recently, a family of secreted bacterial proteins has been implicated in the establishment of parasitic, commensal, and symbiotic prokaryotic-eukaryotic interactions (3, 4). These proteins share sequence and structural homology with the cysteine proteases from adenovirus and the ubiquitin-like protein proteases from eukaryotes. One example is the *Yersinia* effector YopJ that has been shown to block the innate immune response in infected mammalian cells (macrophages) (5). YopJ inhibits the activation of mitogen-activated protein kinase (MAPK)[3] kinases and the IκB kinases (IKK), resulting in inhibition of MAPK and NF-κB signaling pathways (6). Intriguingly, the AvrBsT homolog found in phytopathogenic *Xanthomonas campestris* mediates an "avirulence" function. Specifically, expression of AvrBsT in infected plant cells elicits a host defense (hypersensitivity) response that results in local apoptosis of infected cells and serves to limit further infection by the invading organism (7). Finally, a homolog, Y4LO, from *Rhizobia* sp. may be instrumental in the establishment of a long-term symbiotic state by modulating, rather than disrupting, host defense responses in the root nodule cells of leguminous plants (8).

AvrA, a member of the YopJ/Avr family of proteins (3), exists in most *Salmonella*, however previous studies did not establish a role for this protein (9, 10). Based on our previous observations with NF-κB-inhibitory *Salmonella*, we hypothesized that AvrA was the effector responsible for the observed inhibition of NF-κB signaling.

Materials and Methods

Bacterial strains and construction of mutants

S. typhimurium PhoP^c (1), SL3201, and *S. pullorum* were cultured and used to colonize epithelial cells as previously described (2). *Salmonella* strains carrying a nonpolar mutation in *avrA* were constructed by ligation of a 2 kbp *Eco*RV fragment from pUI1637 (11) conferring resistance to kanamycin into the unique *Bst*BI site of *avrA* after a fill-in reaction with Klenow. The disrupted allele was introduced into the *Salmonella* chromosome by homologous recombination using the pGP704 suicide plasmid (12). The chromosomal configuration of kanamycin-resistant strains was confirmed by Southern hybridization analyses using a nonisotopic detection kit (Roche, Indianapolis, IN) with an *avrA* probe. *avrA* appears to be encoded as a single transcriptional unit, and thus, disruption should not have polar effects on the expression of downstream genes (10).

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[3] Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; IKK, I-κB kinase; CAT, chloramphenicol acetyl transferase; MEKK, MAPK/extracellular signal-regulated kinase kinase kinase; PI, propidium iodide; RT, reverse transcription; qRT-PCR, quantitative RT-PCR.

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Transient transfections and reporter assays

HeLa cells (40–60% confluent) were transiently transfected using Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions and were allowed 16–24 h for expression. DNA quantities were as follows: for chloramphenicol acetyl transferase (CAT) reporter assay, 2 μg pIL-8-CAT, 0–100 ng vector (pCMV-*myc* or pSFFV) and/or expression plasmid per well in a 6-well plate; for immunofluorescence studies, 0.5 μg pCMV-*myc*-AvrA or AvrAC186A per 12-mm coverslip; for Western blot, 2 μg each pIκB-α-FLAG and pCMV5-ΔMAPK/extracellular signal-regulated kinase kinase kinase (MEKK)1-HA, 250 ng each AvrA, YopJ, AvrA/C186A, YopJ/C172A expression plasmids. Transfections of 293 cells were performed as previously described (6). DNA quantities were as follows: 40 ng pNF-κB-Luc (Stratagene, La Jolla, CA), 25 ng pcDNA-p65, 50 ng pCMV5-ΔMEKK1-HA, 400 ng pcDNA-IKK-β, and 20, 100, and 40 ng of pCMV-*myc*-AvrA or pSFFV-YopJ-Flag with p65, MEKK or IKK, respectively. pCMV-*lacZ* (200 ng) was included in some experiments to normalize results for transfection efficiency. CAT (13), luciferase, and β-galactosidase activities (6) were measured as described previously.

Immunofluorescence studies

Immunofluorescent labeling of transiently transfected adherent HeLa cells grown on 12-mm glass coverslips was performed as follows: cells were fixed for 20 min in 3.7% paraformaldehyde in PBS, washed in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and washed again. Fixed samples were incubated in blocking solution (5% normal goat serum in PBS) overnight at 4°C. A 1-hr incubation with each Ab diluted in blocking buffer followed: 1/500 rabbit anti-p65 (Rockland, Gilbertsville, PA); 1/200 fluorescein (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA); 1/300 c-Myc monoclonal (Clontech Laboratories, Palo Alto, CA); 1/300 rhodamine (tetramethylrhodamine isothiocyanate)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Cells were washed three times between each Ab. The coverslips were mounted on glass slides and stained cells were observed by laser confocal epifluorescence microscopy (Zeiss, Oberkochen, Germany).

Western blot analysis

Cell lysates were prepared, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose as previously described (2). Immunoreactive proteins were detected with Abs to IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-IκB-α (Cell Signaling Technology, Beverly, MA), MEKK1 (Santa Cruz Biotechnology), or c-*myc* (Clontech Laboratories) using ECL (Amersham, Piscataway, NJ) and a HRP-conjugated donkey anti-rabbit secondary Ab. Blots were exposed to film for 1–5 min.

Apoptosis assays

After 18–24 h of treatment, 1×10^6 adherent HeLa cells were trypsinized and incubated with FITC-conjugated annexin V (binds to phosphatidyl serine on the cytoplasmic surface of the cell membrane) and propidium iodide (PI) for 15 min in the dark according to the manufacturer's protocol (Annexin V^{FITC} Apoptosis Detection kit; Oncogene Research Products, San Diego, CA). Cells were analyzed by flow cytometry.

Real-time quantitative RT-PCR (qRT-PCR) analysis of IL-8 mRNA

Total RNA was extracted from treated HT-29 cells using TRIzol reagent and reverse-transcribed using a commercial kit (TaqMan Reverse Transcription (RT) kit; PerkinElmer, Boston, MA) according the manufacturer's directions. The RT cDNA reaction products were subjected to real-time quantitative PCR (SYBR Green PCR Core kit; PerkinElmer) with primers for IL-8 (Invitrogen, Carlsbad, CA) and 18s ribosomal RNA (PerkinElmer) as previously described (14). The IL-8 expression level was normalized to the 18s rRNA level of the same sample. Fold difference was the ratio of the normalized value of each sample to that of uninfected control cells. All PCR were performed in triplicate.

Results

We cloned and sequenced the *avrA* gene from wild-type *S. typhimurium* strain SL3201. Sequence analysis revealed that the *avrA* allele used in this study is identical to the allele from *S. typhimurium* LT2 (GenBank accession no. AE008830), but differs from the allele used in previous studies (GenBank accession no. AF013573) (10) by the addition of three thymine nucleotides that result in an additional lysine residue at amino acid position 154. When AvrA was expressed in transiently transfected HeLa epithelial cells, it potently inhibited TNF-α-induced activation of a NF-κB-dependent IL-8-CAT reporter in a dose-dependent fashion (Fig. 1*a*). Expression of a mutant AvrA protein with a single amino acid residue transition (AvrA/C186A) in a putative catalytic cysteine of this enzyme did not prevent TNF-α-stimulated induction of the reporter. Similarly, the corresponding catalytically inactivated mutant YopJ/C172A (15) had no effect, while wild-type YopJ mediated a repressive effect similar to that of wild-type AvrA (Fig. 1*b*).

In addition, we used immunolocalization of the p65 subunit of NF-κB to study activation of this signaling pathway. As previously shown, TNF-α induces cytoplasmic to nuclear translocation of p65 (2) (Fig. 1*c*). However, expression of AvrA-*myc* in HeLa epithelial cells prevented the nuclear translocation of p65 in response to TNF-α (Fig. 1*c*, arrowheads), while mutant AvrA/C186A did not block p65 translocation. Collectively, these results indicate that AvrA is sufficient to inhibit the NF-κB pathway, presumably by an enzymatic activity similar to that of homologous ubiquitin-like protein proteases in other animal and plant pathogens (15).

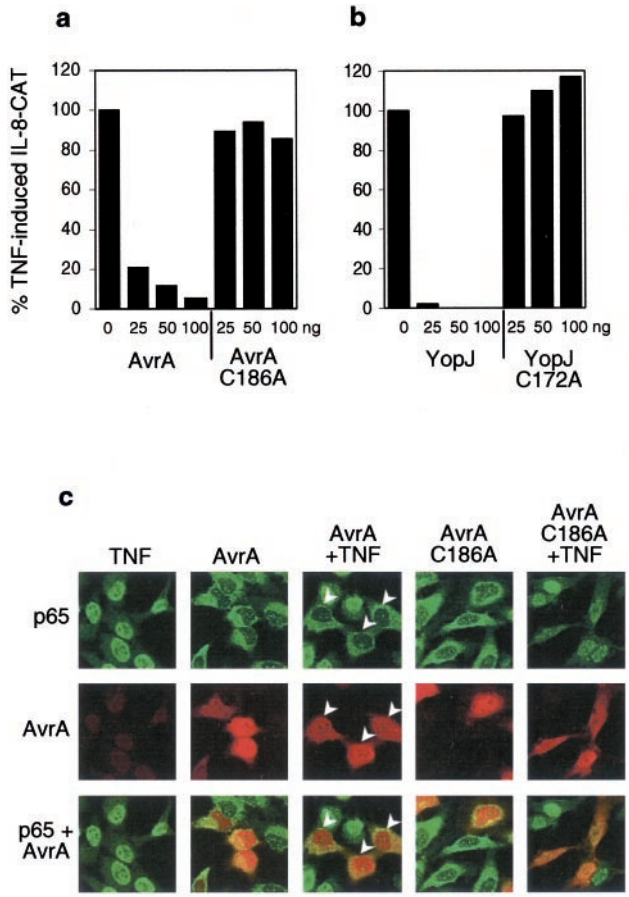

FIGURE 1. AvrA inhibits activation of NF-κB. *a* and *b*, CAT assay of extracts from HeLa cells cotransfected with a NF-κB-dependent pIL-8-CAT reporter, vectors pCMV-*myc* or pSFFV, and expression plasmids for AvrA, AvrA/C186A, YopJ, or YopJ/C172A at the doses indicated, and stimulated with TNF-α (10 ng/ml) for 8 h (13). Results are expressed as a percent of the CAT activity induced by TNF (100%). Experiments with a synthetic NF-κB promoter-CAT reporter produced similar results to those shown in this study. *c*, Immunofluorescent labeling of p65 (green, *top* and *bottom panels*) and *myc*-AvrA (red, *middle* and *bottom panels*) in epithelial cells. Adherent HeLa cells were transfected with pCMV-*myc*-AvrA or pCMV-*myc*-AvrA/C186A expression plasmids and stimulated with TNF-α (10 ng/ml) for 30 min. Arrowheads (*middle panels*) mark cells transfected with AvrA that inhibit translocation of p65 from the cytoplasm into the nucleus.

To identify the point of disruption of the NF-κB pathway by AvrA, we performed a biochemical epistasis assay. 293 cells were transfected with a NF-κB-dependent reporter gene plasmid and plasmids for expression of p65, IKK-β, or an activated form of MEKK1, and AvrA or YopJ. In this assay, MEKK1, IKK-β, and p65 were used to activate gene transcription via the NF-κB pathway and the effects of equivalent amounts of AvrA and YopJ were assessed. Neither AvrA nor YopJ inhibited expression of the reporter gene when stimulated by p65 (Fig. 2*a*), indicating that inhibition of the NF-κB pathway mediated by both AvrA and YopJ occurs at a step that is proximal to p65 nuclear translocation and transcriptional activation. In contrast, both AvrA and YopJ inhibited expression of the reporter gene when stimulated by MEKK1, the MAPK kinase kinase responsible for phosphorylation of IKK (Fig. 2*b*). Finally, when cells were stimulated by the physiologic, proinflammatory IKK-β, AvrA inhibited reporter gene expression while YopJ had little effect (Fig. 2*c*). Thus, AvrA blocks the NF-κB pathway downstream of IKK activation while YopJ blocks at a point upstream of IKK activation (Fig. 2*d*). These data show that although these two molecules are 86% similar in amino acid sequence, they are working, mechanistically, in distinct manners to block NF-κB signaling.

To further address this issue, we assessed the effect of AvrA and YopJ on IκB-α phosphorylation. HeLa cells were transfected with plasmids expressing IκB-α-FLAG, MEKK1 (as the activator of endogenous IKK), and wild-type or mutant AvrA or YopJ. Strikingly, Western blotting of cell lysates revealed that while expression of AvrA had relatively little effect on phosphorylation of IκB-α, expression of wild-type YopJ prevented the appearance of phospho-IκB-α (Fig. 2*e*). This data is consistent with our previous observation that NF-κB-inhibitory *Salmonella* do not block IκB-α phosphorylation in colonized epithelial cells (2). It also correlates with a previous result that YopJ blocks the activation of the superfamily of MAPK kinases (6) and the above observation that AvrA is working distal to this point.

Endogenous and exogenous inhibitors of the NF-κB pathway are potently proapoptotic (16). AvrA null mutants were constructed by disrupting the chromosomal *avrA* gene in strains of *Salmonella* that exhibited the NF-κB inhibitory phenotype, *S. typhimurium* PhoP^c or *S. pullorum*, and were analyzed for the ability to modulate epithelial apoptotic and proinflammatory pathways. Colonization of HeLa epithelial cells for extended periods by PhoP^c or *S. pullorum* strains initiated apoptosis in 9–16% of the total intact cell population as measured by flow cytometry of annexin V- and PI-stained cells (Fig. 3, *a* and *e*). In contrast, basal levels of apoptosis similar to an uninfected control (3–6% of intact population) are observed when cells are colonized by the AvrA null mutants under the same conditions (Fig. 3, *b*, *f*, *d*, and *h*). In a complementation experiment, colonization by AvrA null strains expressing AvrA from a plasmid in *trans* induced levels of apoptosis (13–17% of cells) that were equal to or greater than that observed with PhoP^c or *S. pullorum* (Fig. 3, *c* and *g*).

We also assessed the effects of NF-κB-inhibitory and -derived AvrA mutant strains on direct proinflammatory signaling events such as IL-8 expression. HeLa cells were transiently transfected with a NF-κB-dependent IL-8 promoter-CAT reporter plasmid and CAT expression was measured in colonized cells. As previously shown, TNF-α induces significant CAT expression, but colonization with *S. pullorum* represses ~90% of this activation (Fig. 4*a*) (2). The *S. pullorum* AvrA mutant repressed only 55% of TNF-α-stimulated CAT expression and, therefore, has partially lost the ability to block proinflammatory signaling. In addition, IL-8 expression levels are higher in HT-29 epithelial cells colonized with AvrA mutant strains than in cells colonized with the parent inhibitory strains PhoP^c or *S. pullorum* (Fig. 4*b*) as demonstrated by real-time qRT-PCR. Together, these data reveal that AvrA is a significant contributor in the observed inhibition of proinflammatory signaling by NF-κB-inhibitory *Salmonella* strains.

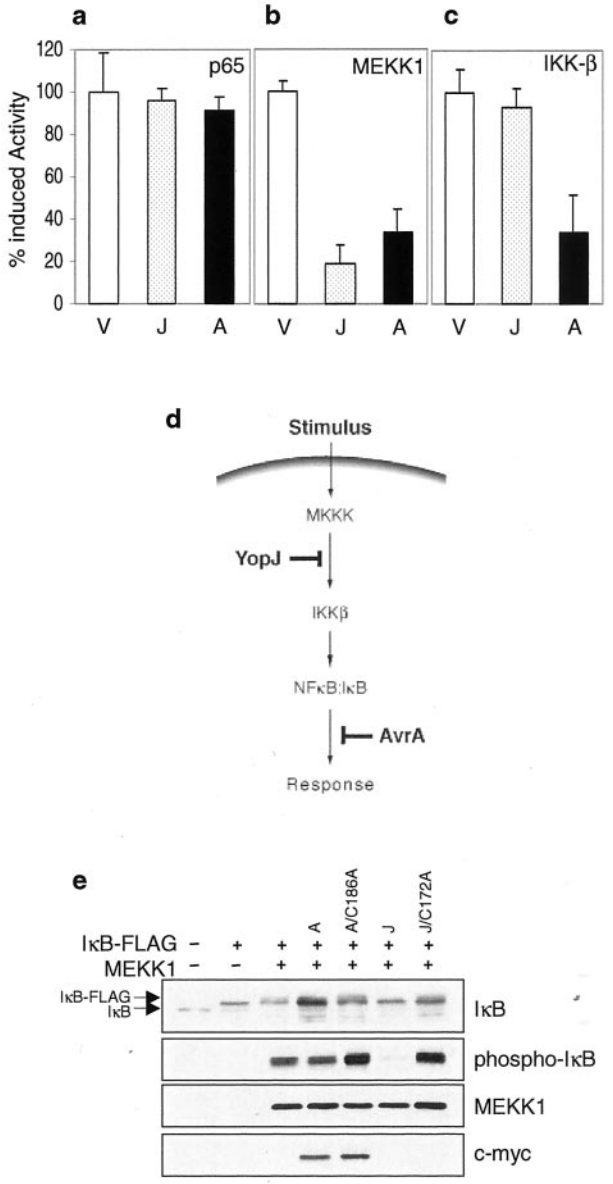

FIGURE 2. AvrA inhibits the NF-κB pathway downstream of IKK-β. *a–c*, Luciferase assay of extracts from 293 cells cotransfected with a NF-κB-Luc reporter plasmid, pCMV-*lacZ* plasmid, expression plasmids for p65, MEKK1, or IKK-β (as inducers), and AvrA (A), YopJ (J), or vector (V) as indicated. Data are the mean of three experiments and are shown as percent induced activity. β-Galactosidase activity from pCMV-*lacZ* was assayed and used to normalize all reactions for transfection efficiency. *d*, A diagram of the inhibition of the NF-κB pathway by YopJ and AvrA. YopJ blocks MAPK kinase kinase (MKKK) activation of IKK-β. AvrA blocks the pathway downstream of IKK-β. *e*, Immunoblots showing that YopJ inhibits IκB-α phosphorylation, but AvrA does not. Cells were transfected with plasmids expressing IκB-α-FLAG, MEKK1 (as an inducer), and wild-type or mutant AvrA (A and A/C186A, respectively) or YopJ (J and J/C172A, respectively), allowed 18 h for expression, and incubated with 250 nM MG-262 (proteasome inhibitor; Affiniti, Exeter, U.K.) for 4 h. Cell lysates were prepared and blotted with the Abs indicated on the *right*. Note the difference in size of endogenous IκB (*bottom arrow*) and IκB-FLAG (*top arrow*).

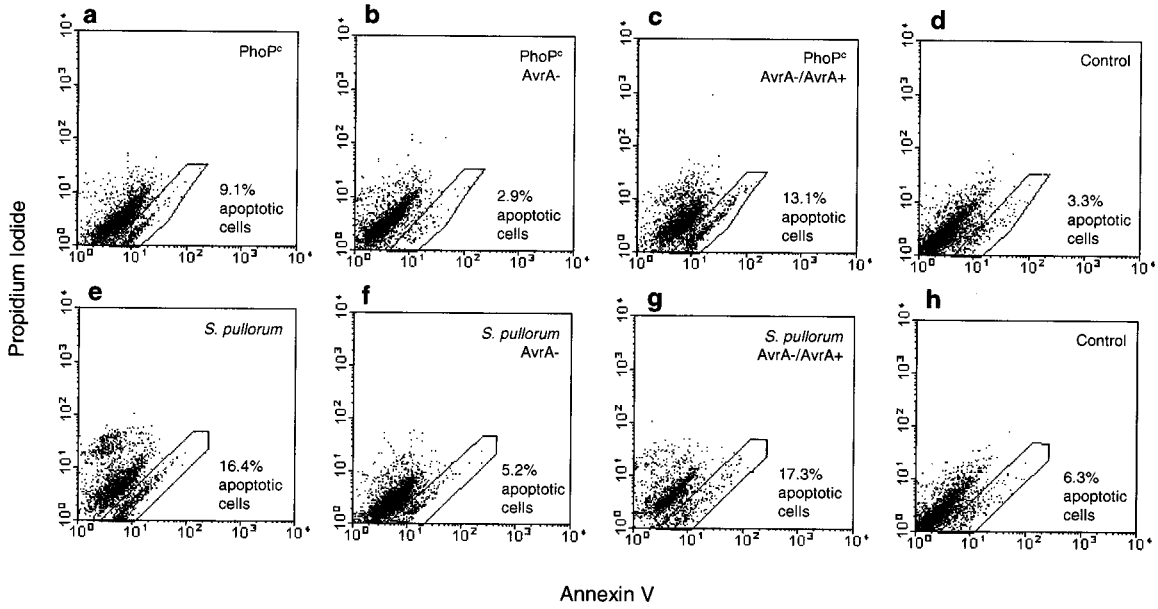

FIGURE 3. AvrA elicits apoptosis of HeLa cells infected with *Salmonella. a–h,* Partially confluent monolayers of HeLa cells were colonized with the indicated *Salmonella* strains ($AvrA^-$, mutant strain; $AvrA^-/AvrA^+$, mutant strain complemented with a plasmid expressing AvrA; control, uninfected) for 1 h, washed, and further incubated for 24 h in complete DMEM with gentamicin (50 μg/ml) to inhibit growth of extracellular bacteria. Adherent and nonadherent cells were pooled, stained with Annexin V^{FITC} and PI, and analyzed by flow cytometry. Early apoptotic cells (population of interest) have increased Annexin V^{FITC} fluorescence only, whereas necrotic and late apoptotic cells have increased annexin V and PI fluorescence. Dot plots of the intact (live) cell population show increased Annexin V^{FITC} fluorescence (*x*-axis), indicating increased apoptosis, in a subpopulation of cells infected by strains containing AvrA (gated population). The percentages of apoptotic cells within the gated areas are indicated. Axes are labeled with arbitrary fluorescence units. Data are from a representative of three independent experiments that gave similar results.

Discussion

In this report, we show that AvrA mediates inhibition of the NF-κB pathway in cultured human epithelia and results in consequent activation of apoptosis. Our data indicates that AvrA and YopJ, two similar molecules presumably involved in the metabolism of ubiquitin or related molecules, have evolved to inhibit the NF-κB pathway at separate points. As we have shown, YopJ is a potent inhibitor of IκB-α phosphorylation in vivo and in vitro (6), while AvrA is not, indicating different points of action. This observation underscores the crucial, multifaceted role that modification by ubiquitin-like molecules plays in the NF-κB pathway, and perhaps in other stress-induced signaling pathways. At least two ubiquitin modification events are critical in the activation of the NF-κB pathway: the typical Lys^{48}-linked polyubiquitination of IκB necessary for proteasomal degradation (17), and the Lys^{63}-linked ubiquitination of TNFR-associated factor 6 necessary for IKK catalytic activation (18). Furthermore, modification of the Cul subunit of the Skp1/Cul/F-box ubiquitin ligase complex by the ubiquitin-like molecule Nedd-8 has been shown to be necessary for IκB degradation (19). Neither recombinant AvrA nor YopJ are able to hydrolyze in vitro ubiquitinated IκB-α (data not shown), suggesting that these proteins do not directly deubiquitinate IκB-α. It is possible that YopJ inhibits proximal events such as TNFR-associated factor 6 modification, while AvrA may influence a more distal event, such as Skp1/Cul/F-box ubiquitin ligase activation.

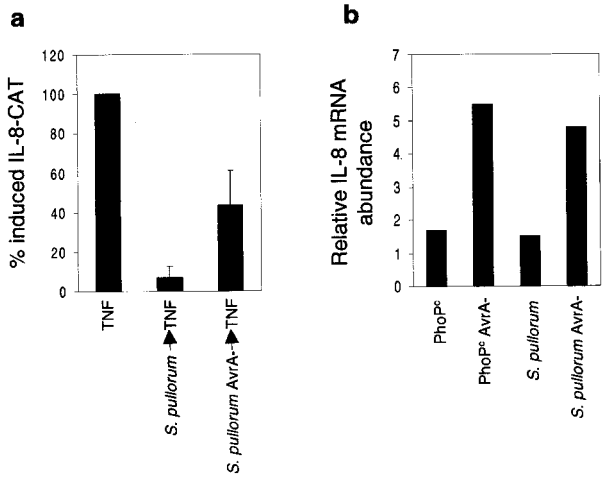

FIGURE 4. Disruption of *avrA* partially relieves the inhibition of proinflammatory signaling by NF-κB-inhibitory *Salmonella. a,* CAT assay of extracts from HeLa cells transfected with a NF-κB-dependent IL-8-CAT reporter and colonized with the indicated bacteria for 1 h, followed by TNF-α challenge for 8 h in the presence of gentamicin (50 μg/ml) to prevent bacterial proliferation. CAT was measured by ELISA. Data are the mean of three experiments and are shown as a percent of TNF-induced CAT activity. *b,* Real-time qRT-PCR analysis of colonization-associated changes in IL-8 mRNA levels in *Salmonella*-infected HT-29 epithelial cells. Cells were colonized with the indicated bacteria for 1 h, washed, and incubated 2 h longer. Values are expressed relative to IL-8 mRNA levels in uninfected HT-29 cells. For all reactions, mRNA was assayed in triplicate and data shown are from a representative of three independent experiments that yielded similar results.

Bacterial effectors of the ubiquitin-like protein protease family are believed to exert their function by initiating or augmenting apoptosis, presumably by inhibition of NF-κB or related anti-apoptotic pathways (3). In certain *Salmonella* infections, the presence of AvrA in the infected epithelia would result in accelerated apoptosis, allowing elimination of the infected cells and prevention of systemic spread. As epithelial cells are rapidly replaced, having a crypt-to-villus life span of 3–5 days (20), apoptosis-mediated cell death would not have deleterious systemic consequences (as would be the case with loss of macrophages). Significantly, *Salmonella typhi* and *paratyphi* are strains that evade epithelial defenses and

result in severe systemic disease (within macrophages). These strains invariably do not possess an *avrA* allele (21). Consistently, Grassme et al. (22) reported that in vivo murine airway infection by *Pseudomonas aeruginosa* results in apoptosis of respiratory epithelial cells and limitation of further infection. In contrast, respiratory infection in mice genetically deficient in the CD95/CD95-ligand apoptotic activation pathway results in systemic dissemination of the airway pathogen and rapid death. The observations described in this study support the hypothesis that the mammalian host may exploit apoptosis of rapidly turned over epithelial cell types as a defense mechanism. This strategy is functionally analogous to the interaction of plant pathogen Avr homologs with the R genes of resistant plants (23) in that the recognition of infection causes induction of the host defense response. These observations shed new light on mechanisms of pathogenic, commensal, and even symbiotic relationships between a eukaryotic host and associated prokaryotes.

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