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BACTERIAL INFECTIONS

Redundant and Cooperative Roles for *Yersinia pestis* Yop Effectors in the Inhibition of Human Neutrophil Exocytic Responses Revealed by Gain-of-Function Approach

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ABSTRACT *Yersinia pestis* causes a rapid, lethal disease referred to as plague. *Y. pestis* actively inhibits the innate immune system to generate a noninflammatory environment during early stages of infection to promote colonization. The ability of *Y. pestis* to create this early noninflammatory environment is in part due to the action of seven Yop effector proteins that are directly injected into host cells via a type 3 secretion system (T3SS). While each Yop effector interacts with specific host proteins to inhibit their function, several Yop effectors either target the same host protein or inhibit converging signaling pathways, leading to functional redundancy. Previous work established that *Y. pestis* uses the T3SS to inhibit neutrophil respiratory burst, phagocytosis, and release of inflammatory cytokines. Here, we show that *Y. pestis* also inhibits release of granules in a T3SS-dependent manner. Moreover, using a gain-of-function approach, we discovered previously hidden contributions of YpkA and YopJ to inhibition and that cooperative actions by multiple Yop effectors are required to effectively inhibit degranulation. Independent from degranulation, we also show that multiple Yop effectors can inhibit synthesis of leukotriene B_4 (LTB_4), a potent lipid mediator released by neutrophils early during infection to promote inflammation. Together, inhibition of these two arms of the neutrophil response likely contributes to the noninflammatory environment needed for *Y. pestis* colonization and proliferation.

KEYWORDS *Yersinia pestis*, granules and degranulation, leukotriene, neutrophils, plague, type 3 secretion

Plague is the human disease caused by infection with the bacterial pathogen *Yersinia pestis* (1). Depending upon the route of inoculation, plague can manifest in three forms (1). Primary bubonic, pneumonic, or septicemic plague arises when bacteria are inoculated into the skin, lungs, or bloodstream, respectively. Upon infection with *Y. pestis*, mean time to death without medical intervention can range from 3 days for primary pneumonic or septicemic plague to 7 days for bubonic plague. A hallmark of *Y. pestis* infection is the lack of inflammation during early stages of colonization. During pneumonic plague in mice, a minimal inflammatory response is observed for the first 24 to 36 h of infection (2–5). Beginning at ~48 h postinfection, the inflammatory response to *Y. pestis* changes, resulting in a significant increase in inflammatory mediators, including monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNF-α), interleukin 12p70 (IL-12p70), gamma interferon (IFN-γ), and IL-6 (2,

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5). This coincides with an influx of immune cells, especially neutrophils, into the lungs, resulting in a rapid pneumonia (2, 5). Similarly, inflammation is delayed in bubonic plague and does not occur until after *Y. pestis* has begun to proliferate in the draining lymph node and disseminate (6–8). The ability of *Y. pestis* to actively inhibit innate immune responses is a key virulence mechanism for *Y. pestis* (2, 3, 5, 6, 8, 9).

Normally, neutrophils are recruited in response to a variety of stimuli derived from damaged or activated host cells via damage-associated molecular patterns (DAMPs), cytokines, chemokines, or complement products (10–12). Microbial components, such as lipopolysaccharide, peptidoglycan, or *N*-formylmethionine-leucyl-phenylalanine peptides (fMLF), known as pathogen-associated molecular patterns (PAMPs), can also stimulate the recruitment of neutrophils (13). Upon stimulation, neutrophils traverse the vasculature to reach the site of infection. Upon arrival at the site of infection, neutrophil antimicrobial responses are multifactorial and are comprised of phagocytosis, induction of the respiratory burst, degranulation, and release of neutrophil extracellular traps (NETs) (13). Combined efforts from each of these responses make neutrophils very adept at killing microorganisms. Phagocytosis is important for clearing many bacterial infections, although some pathogens have acquired virulence factors that inhibit uptake by neutrophils (14, 15). In such situations, neutrophils rely upon extracellular release of antimicrobial mechanisms to effectively clear the infection.

One mechanism utilized by neutrophils to combat extracellular pathogens is the release of antimicrobial cargo contained in preformed granules (a process referred to as degranulation or graded exocytosis) (16). Degranulation occurs in a regulated manner to coordinate release or modification of cytokines, chemokines, and signaling ligands/receptors to facilitate neutrophil transmigration and chemotaxis with release of antimicrobial components that can directly restrict pathogen growth. Neutrophils contain four different granule subtypes, and mobilization of each granule is tightly controlled and dependent on the intensity of stimulation to coordinate functional responses (17). Neutrophil degranulation is hierarchical, with secretory vesicles being the first subtype to undergo exocytosis, followed by gelatinase granules. Degranulation of specific and azurophilic granules, both loaded with toxic antimicrobial cargo, is more limited and requires stronger stimulation to promote granule mobilization (13). Tightly graded control of granule release ensures that contents are released at the correct location to diminish collateral damage to the host.

The ability of neutrophils to mediate inflammatory responses has become more appreciated (18). Neutrophils release a variety of cytokines and chemokines, as well as other immune modulatory factors that contribute to the cellular communication network during inflammation (19, 20). One of the most potent modulators released by neutrophils is leukotriene B_4 (LTB_4). Not only is it important for recruitment of additional neutrophils to the site of infection (21–23), but LTB_4 also enhances the antimicrobial responses of both neutrophils and macrophages, including phagocytosis, respiratory burst, degranulation, and the release of inflammatory cytokines (24–28). Importantly, LTB_4 production is not dependent on transcriptional regulation (29). Therefore, LTB_4 is produced more rapidly than other chemoattractants, such as IL-8, and it is pivotal in mounting a swift inflammatory response (26, 28, 30–32). Moreover, release of LTB_4 is independent of degranulation (33), suggesting that regulation of LTB_4 release also differs from degranulation.

Although neutrophils are extremely capable of restricting microbial colonization, *Y. pestis* encodes a variety of virulence factors to evade recognition and killing by neutrophils (34–38). The Ysc type 3 secretion system (T3SS) secretes seven *Yersinia* outer protein (Yop) effectors directly into host cells and is paramount for inhibition and evasion of neutrophil responses (4, 9, 34, 39–43). Moreover, several *in vivo* studies have demonstrated that neutrophils are the primary cell type that *Y. pestis* interacts with during early stages of infection (4, 36, 44). Once injected into neutrophils, *Y. pestis* Yop effectors interact with specific host factors to disrupt multiple host signaling pathways. YpkA, YopE, YopH, and YopT disrupt the actin cytoskeleton via interactions with host Rac, Rho, and focal adhesion complex proteins (41–43, 45–59). YopH has also been

shown to inhibit host cell calcium flux (49, 60), while YopJ inhibits mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) cascades (40, 60–63). Together, these Yop effectors have been shown to effectively inhibit neutrophil phagocytosis, respiratory burst, and cytokine/chemokine release (34, 40, 61, 63). Importantly, the Yop translocon pore and effects of Yop effectors on host proteins can trigger inflammasome activation, which should lead to inflammatory responses (64–67). However, YopM and YopK function to inhibit inflammasome activation and subsequent inflammatory responses (64, 65, 67–72). Together, the Yop effectors allow *Y. pestis* to actively inhibit the inflammatory response.

Recently it was shown that *Yersinia pseudotuberculosis* inhibits neutrophil degranulation in a T3SS-dependent manner, which was dependent on the actions of YopE and YopH (73). Here, we show T3SS-dependent inhibition of neutrophil degranulation by *Y. pestis*, as well as roles of both YopE and YopH in inhibition. However, using a gain-of-function approach with a library of *Y. pestis* strains only expressing one Yop effector, we were able to identify additional Yop effectors contributing to inhibition of degranulation that have not been previously observed. Moreover, we show for the first time that *Y. pestis* actively inhibits production of LTB_4 by human neutrophils, and we identify the Yop effectors contributing to this inhibition.

RESULTS

***Y. pestis* inhibits neutrophil degranulation in a T3SS-dependent manner.** Degranulation is a highly regulated but quick response that generally occurs within minutes after encountering a stimulus. Multiple studies have provided an understanding of the contents of the different granules that are released during degranulation (e.g., albumin is released during degranulation of secretory vesicles; gelatinase is released during degranulation of gelatinase granules), and the increased expression of receptors displayed on the neutrophil cell surface upon granule fusion with the plasma membrane (e.g., CD66b is displayed after degranulation of specific granules; CD63 is displayed after degranulation of azurophilic granules) (reviewed by Cowland and Borregaard [74]). Importantly, using these markers, degranulation of each granule subtype in response to different stimuli can be reliably monitored. Recently, it has been shown that *Y. pseudotuberculosis* inhibits degranulation by human neutrophils (73). To determine whether *Y. pestis* similarly inhibits degranulation, human neutrophils were infected with *Y. pestis* CO92 or with a strain lacking the pCD1 plasmid encoding the Ysc T3SS [*Y. pestis* CO92 T3$^{(-)}$]. At a multiplicity of infection (MOI) of 10 or 100, minimal, if any, release of the four granule subtypes was observed in response to *Y. pestis* CO92 (Fig. 1). Similarly, at an MOI of 10, infection with *Y. pestis* CO92 T3$^{(-)}$ did not result in degranulation. However, at an MOI of 100, *Y. pestis* CO92 T3$^{(-)}$ caused significant release of all four granule subtypes compared to infection with *Y. pestis* CO92 (Fig. 1 and Fig. S1A and B). Infection with a *Y. pestis* KIM derivative with and without the pCD1 plasmid recapitulated the phenotypes observed for *Y. pestis* CO92 and CO92 T3$^{(-)}$, respectively. Together, these data indicate that degranulation is inhibited by *Y. pestis* in a T3SS-dependent manner.

Cooperative inhibition of neutrophil degranulation by Yop effectors revealed through gain-of-function approach. The Ysc T3SS delivers seven effector proteins into targeted host cells (75). To determine if a single Yop effector is responsible for inhibiting neutrophil degranulation, human neutrophils were infected with a library of *Y. pestis* KIM1001 strains containing in-frame deletions of one *yop* gene (Table S1). While *Y. pestis* is able to inhibit release of all four granule subtypes (Fig. 1), specific and azurophilic granules contain most of the antimicrobial components produced by neutrophils, and are typically released at the site of infection, where neutrophils would come into direct contact with *Y. pestis*. We therefore focused on the ability of *Y. pestis* Yop effectors to inhibit release of these two granule subtypes. Moreover, comparing the expression of degranulation markers after incubation with *Y. pestis* T3$^{(-)}$ for 30 and 60 min indicated that degranulation peaked by 30 min postinfection (Fig. S1C and D). Therefore, degranulation was monitored at 30 min postinfection for subsequent exper-

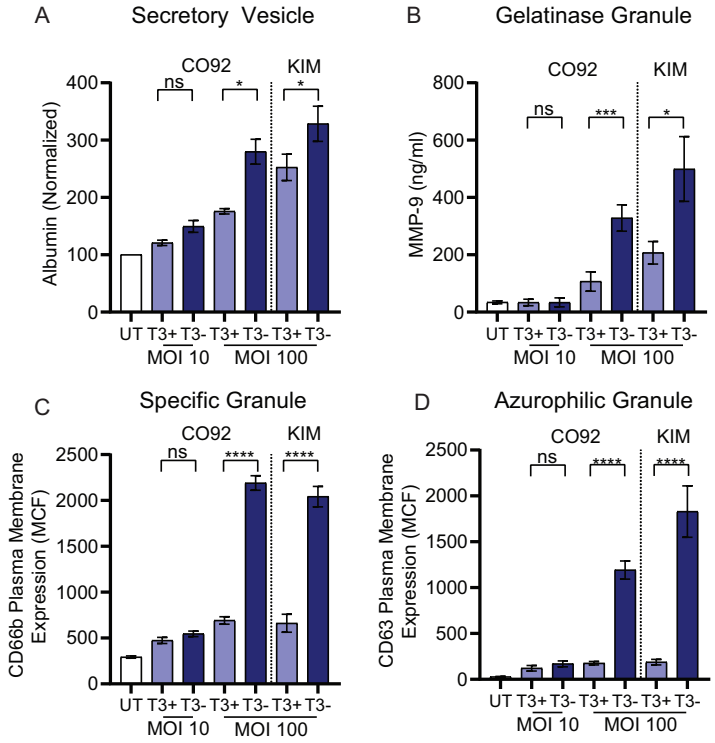

FIG 1 *Y. pestis* inhibits degranulation in a T3SS-dependent manner. Human neutrophils (4×10^6) were infected with *Y. pestis* CO92 or *Y. pestis* KIM1001 with or without the pCD1 plasmid encoding the T3SS ($T3^+$ or $T3^-$, respectively) at indicated multiplicities of infection (MOIs) (10 or 100). Degranulation was measured after 30 min of infection for (A) secretory vesicles, (B) gelatinase, (C) specific, and (D) azurophilic granules. UT, untreated cells. Mean ± standard error of the mean (SEM) from 5 biologically independent experiments. One-way analysis of variance (ANOVA) with Sidak's *post hoc* test; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. Representative flow cytometry histograms for specific and azurophilic granules are shown in Fig. S1.

iments. As shown in Fig. 1, infection with the *Y. pestis* KIM1001 $T3^{(-)}$ strain resulted in significant release of both specific and azurophilic granules compared to infection with *Y. pestis* KIM1001 (Fig. 2). Mutants lacking any single *yop* gene retained the ability to inhibit release of either granule, with surface expression of degranulation markers similar to that observed for *Y. pestis* KIM1001. Similar results were observed with individual *yop* deletion mutants in the *Y. pestis* CO92 background (data not shown). These data suggest that more than one Yop effector protein is able to inhibit neutrophil granule release (i.e., functional redundancy in the system).

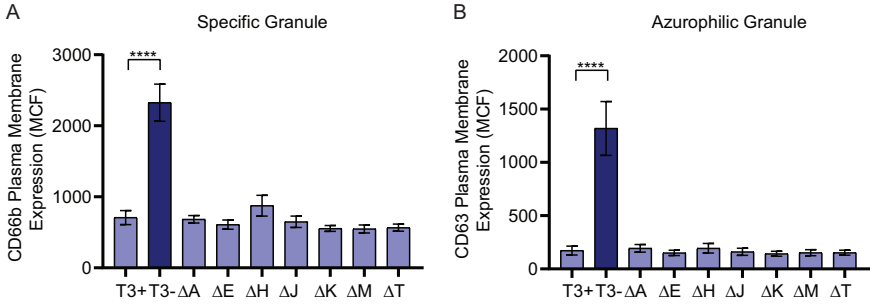

FIG 2 Deletion of individual Yop effector proteins does not alter neutrophil degranulation response to *Y. pestis* infection. Human neutrophils (4×10^6) were infected with *Y. pestis* KIM1001 with or without the pCD1 plasmid encoding the T3SS ($T3^+$ or $T3^-$, respectively) or with strains lacking *ypkA* (ΔA), *yopE* (ΔE), *yopH* (ΔH), *yopJ* (ΔJ), *yopK* (ΔK), *yopM* (ΔM), or *yopT* (ΔT); MOI = 100. Degranulation was measured after 30 min of infection for (A) specific or (B) azurophilic granules. Mean ± SEM from 5 biologically independent experiments. One-way ANOVA with Dunnett's *post hoc* test to $T3^+$; ****, $P < 0.0001$.

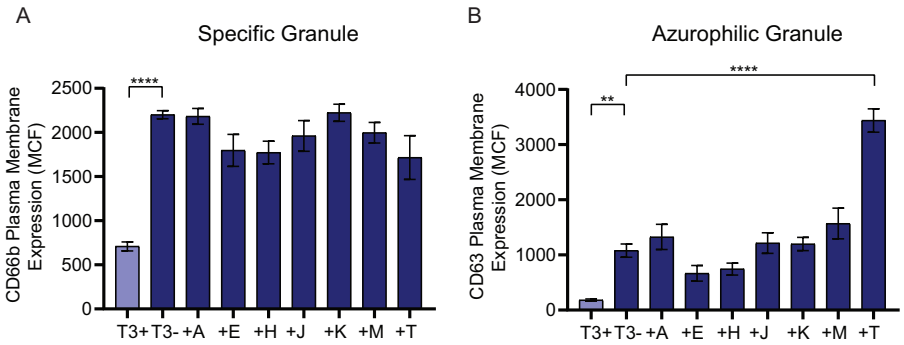

FIG 3 Individual Yop effector proteins are unable to completely inhibit degranulation. Human neutrophils (4×10^6) were infected with *Y. pestis* KIM1001 with or without the pCD1 plasmid encoding the T3SS ($T3^+$ or $T3^-$, respectively) or with strains expressing only *ypkA* (+A), *yopE* (+E), *yopH* (+H), *yopJ* (+J), *yopK* (+K), *yopM* (+M), or *yopT* (+T); MOI = 100. Degranulation was measured after 30 min of infection for (A) specific or (B) azurophilic granules. Mean ± SEM from 5 biologically independent experiments. One-way ANOVA with Dunnett's *post hoc* test to $T3^-$: **, $P < 0.01$; ****, $P < 0.0001$.

Recently, Palace et al. developed a library of *Y. pestis* strains that only express one Yop effector (62). This library allows for the study of individual Yop effectors without the presence of the other six, which could confound data interpretation due to phenotypical masking by functionally redundant proteins. To determine whether individual Yop effectors inhibit degranulation, neutrophils were infected with strains from this library and monitored for exocytosis of specific and azurophilic granules (Fig. 3). While strains expressing YopE, YopH, or YopT trended toward decreased specific granule exocytosis, none of the mutants demonstrated statistically significant decreases in exocytosis compared to the $T3^{(-)}$ strain (Fig. 3A). Similar trends were observed for exocytosis of azurophilic granules for strains expressing YopE and YopH, but surprisingly, the strain expressing only YopT caused increased release of azurophilic granules (Fig. 3B). These data indicate that while there is functional redundancy for inhibiting degranulation by neutrophils, the effector proteins also work in a cooperative manner during *Y. pestis* infection to effectively inhibit exocytosis of specific and azurophilic granules.

YopE, YopH, YopJ, and YpkA act cooperatively to inhibit degranulation of specific and azurophilic granules. To determine which Yop effectors act cooperatively to inhibit degranulation, a coinfection approach with two strains of *Y. pestis* expressing different individual Yop effectors was employed. Neutrophils were infected with a 1:1 mixture of two *Y. pestis* strains, each expressing different Yop proteins (final MOI = 100; MOI of 50 for each strain). Exocytosis of specific and azurophilic granules was compared to that of cells infected with *Y. pestis* KIM1001 $T3^{(-)}$ or a 1:1 mixture of *Y. pestis* KIM1001 and *Y. pestis* KIM1001 $T3^{(-)}$. As expected, coinfections with *Y. pestis* KIM1001 expressing all of the Yop proteins significantly decreased exocytosis of both specific and azurophilic granules compared to infection with only *Y. pestis* KIM1001 $T3^{(-)}$ (Fig. 4 and 5). Coinfection with two strains expressing only one Yop protein revealed that cooperative actions by four effectors were sufficient to inhibit degranulation of both specific and azurophilic granules. Coinfection with strains expressing YopH and YopE, YopH and YpkA, YopH and YopJ, or YopE and YopJ was sufficient to inhibit degranulation of both granules to levels similar to coinfection with *Y. pestis* KIM1001 and *Y. pestis* KIM1001 $T3^{(-)}$ (Fig. 4 and 5). Coinfection with YopH and YopK appeared to also sufficiently inhibit release of azurophilic granules. For specific granules, coinfection with YopH and YopK, YopH and YopT, or YopE and YopT showed intermediate phenotypes. Coinfection with YopT could reverse the ability of YopH and YopE to partially inhibit degranulation of azurophilic granules (Fig. 5A and B), reflecting the enhanced degranulation previously observed in single YopT infection (Fig. 3B). However, coinfection with YopJ or YpkA appeared to inhibit the YopT enhanced degranulation phenotype (Fig. 5C and D). Together, these data confirm previously reported roles for YopH and YopE in

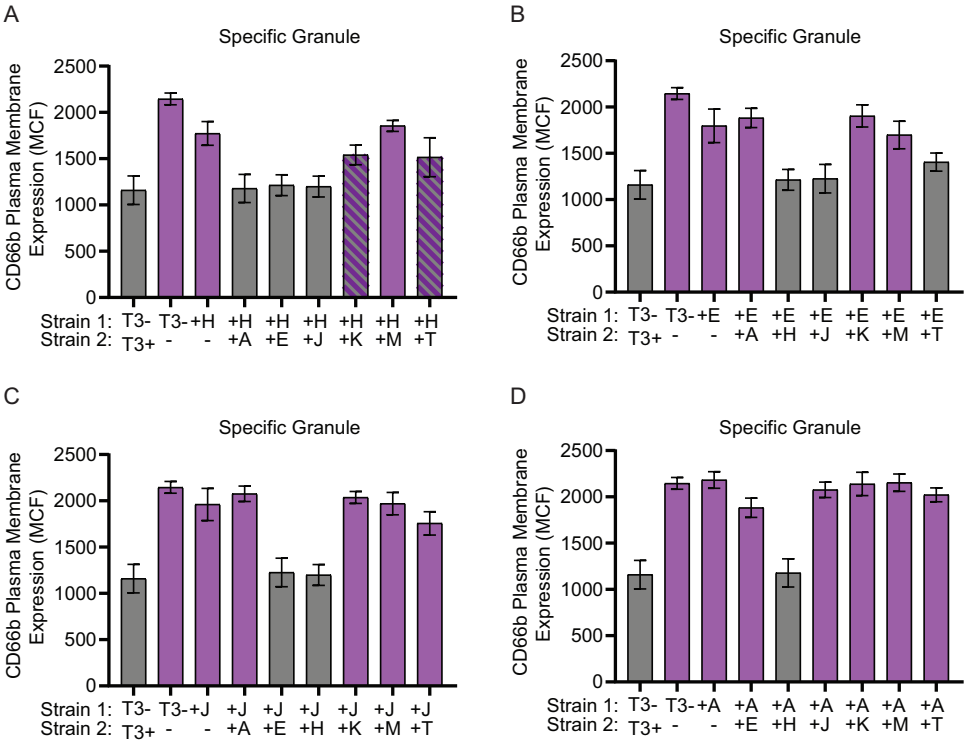

FIG 4 At least two Yop effector proteins are required to fully inhibit specific granule release. Human neutrophils (4×10^6) were coinfected with *Y. pestis* KIM1001 with or without the pCD1 plasmid encoding the T3SS ($T3^+$ or $T3^-$, respectively) or with strains expressing only *ypkA* (+A), *yopE* (+E), *yopH* (+H), *yopJ* (+J), *yopK* (+K), *yopM* (+M), or *yopT* (+T) mixed at a 1:1 ratio with strains expressing only (A) *yopH* (+H), (B) *yopE* (+E), (C) *yopJ* (+J), or (D) *ypkA* (+A); MOI of each strain = 50 for a combined MOI of 100. Specific granule release was measured after 30 min of infection. Mean ± SEM from 5 biologically independent experiments. One-way ANOVA with Dunnett's *post hoc* test. Gray bars are significantly different than $T3^-$ ($P \leq 0.05$); purple bars are significantly different from $T3^-/T3^+$ ($P \leq 0.05$); hatched bars are not significantly different than $T3^-$ or $T3^-/T3^+$.

inhibition of degranulation (73), and also revealed previously hidden contributions of YpkA, YopJ, and YopK.

***Y. pestis* inhibits LTB_4 response of human neutrophils.** LTB_4 is a potent chemoattractant released by neutrophils independent of degranulation, and it contributes to early inflammation in response to infection (22, 33). As inhibition of inflammation is a hallmark of *Y. pestis* infection, we next asked whether *Y. pestis* inhibits release of LTB_4 by human neutrophils. Neutrophils were infected with *Y. pestis* KIM1001 or *Y. pestis* KIM1001 $T3^{(-)}$, and the level of LTB_4 released into the supernatant was compared to that released by untreated neutrophils (Fig. 6A). Infection with *Y. pestis* KIM1001 did not result in significant release of LTB_4 compared to untreated neutrophils. However, when neutrophils were infected with *Y. pestis* KIM1001 $T3^{(-)}$, a significant increase in LTB_4 secretion was observed (Fig. 6A; $P < 0.01$). To determine if these differences in LTB_4 levels were sufficient to alter chemotaxis of naive neutrophils, conditioned supernatants from infected neutrophils were used in a chemotaxis assay and compared to supernatant from untreated neutrophils (Fig. 6B). Naive neutrophils exposed to buffer or fMLF, a known chemoattractant, were used as controls. The numbers of naive neutrophils migrating toward the conditioned supernatant from untreated and *Y. pestis* KIM1001-infected neutrophils were not significantly different. However, in direct correlation with the elevated levels of LTB_4 in the conditioned supernatant, significantly more neutrophils migrated toward the supernatant collected from cells infected with *Y. pestis* KIM1001 $T3^{(-)}$ (Fig. 6B; $P < 0.01$). Pretreatment of naive neutrophils with an inhibitor that blocks signaling through the LTB_4 high-affinity receptor BLT1 eliminated chemotaxis toward the conditioned supernatant but not toward fMLF (Fig. 6C). These

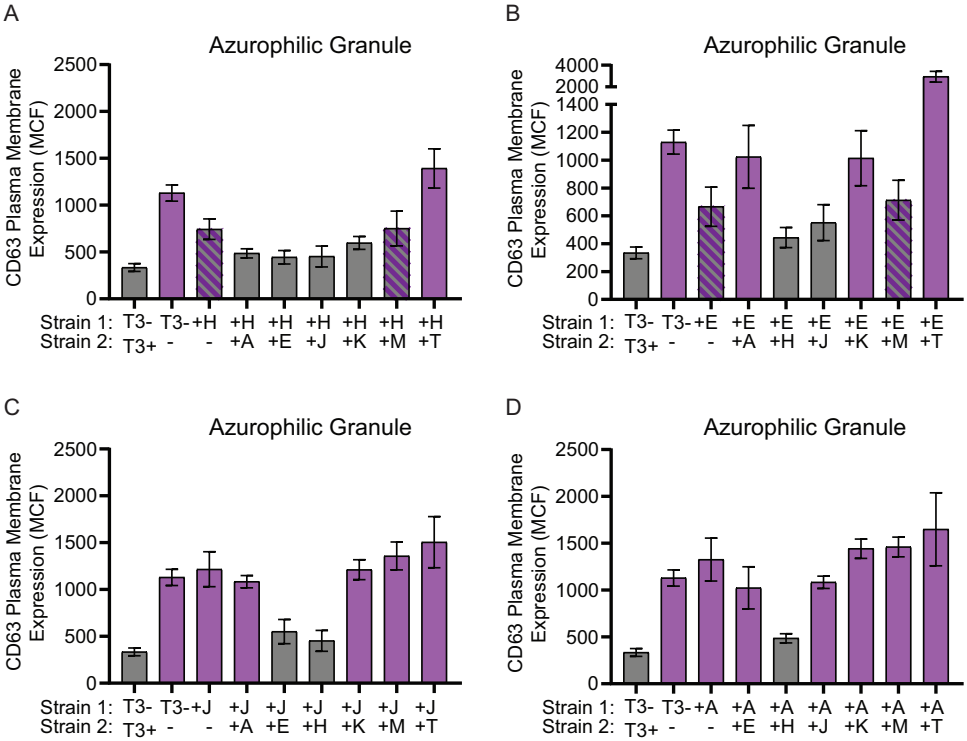

FIG 5 At least two Yop effector proteins are required to fully inhibit azurophilic granule release. Human neutrophils (4×10^6) were coinfected with *Y. pestis* KIM1001 with or without the pCD1 plasmid encoding the T3SS ($T3^+$ or $T3^-$, respectively) or with strains expressing only *ypkA* (+A), *yopE* (+E), *yopH* (+H), *yopJ* (+J), *yopK* (+K), *yopM* (+M), or *yopT* (+T) mixed at a 1:1 ratio with strains expressing only (A) *yopH* (+H), (B) *yopE* (+E), (C) *yopJ* (+J), or (D) *ypkA* (+A); MOI of each strain = 50 for a combined MOI of 100. Azurophilic granule release was measured after 30 min of infection. Mean ± SEM from 5 biologically independent experiments. One-way ANOVA with Dunnett's *post hoc* test. Gray bars are significantly different than $T3^-$ ($P \leq 0.05$); purple bars are significantly different than $T3^-/T3^+$ ($P \leq 0.05$); hatched bars are not significantly different than $T3^-$ or $T3^-/T3^+$.

results indicate that the presence of LTB_4 in the conditioned supernatant was promoting chemotaxis.

Next, we used the library of *Y. pestis* mutants expressing only one Yop effector to ask whether individual effector proteins are sufficient to inhibit LTB_4 release. In contrast to the data observed for inhibition of specific and azurophilic granule release, four of the seven Yop effectors (YpkA, YopE, YopH, and YopJ) were able to inhibit LTB_4 release to levels similar to those of *Y. pestis* KIM1001 (Fig. 6D). Moreover, infection with the strain only expressing YopT also substantially decreased the amount of LTB_4 released from the neutrophils, although to a lesser degree than the other four effectors. Finally, to determine if *Y. pestis* infection inhibits synthesis or release of LTB_4, intracellular levels of LTB_4 from infected neutrophils were measured. Similar to the results observed for conditioned supernatants, significantly lower amounts of intracellular LTB_4 were detected in cells infected with *Y. pestis* KIM1001 and mutants expressing YpkA, YopE, YopH, YopJ, and YopT (Fig. 6E). Together, these data indicate that *Y. pestis* actively inhibits synthesis of LTB_4 from human neutrophils in a T3SS-dependent manner, multiple Yop effectors are sufficient to inhibit LTB_4 synthesis, and the inhibition of LTB_4 release by infected neutrophils negatively impacts the chemotactic activity of naive neutrophils to respond to the infection.

Disruption of the host cytoskeleton inhibits LTB_4 release in response to *Y. pestis* infection. Although different mechanisms are used by YpkA, YopE, YopH, and YopT, all four proteins have been shown to affect actin cytoskeletal rearrangement in host cells (41, 46, 76–78). Because of this common effect, we hypothesized that *Y. pestis* disruption of the actin cytoskeleton could inhibit LTB_4 release. If true, the release of LTB_4 observed during infection with *Y. pestis* KIM1001 $T3^{(-)}$ could be blocked by

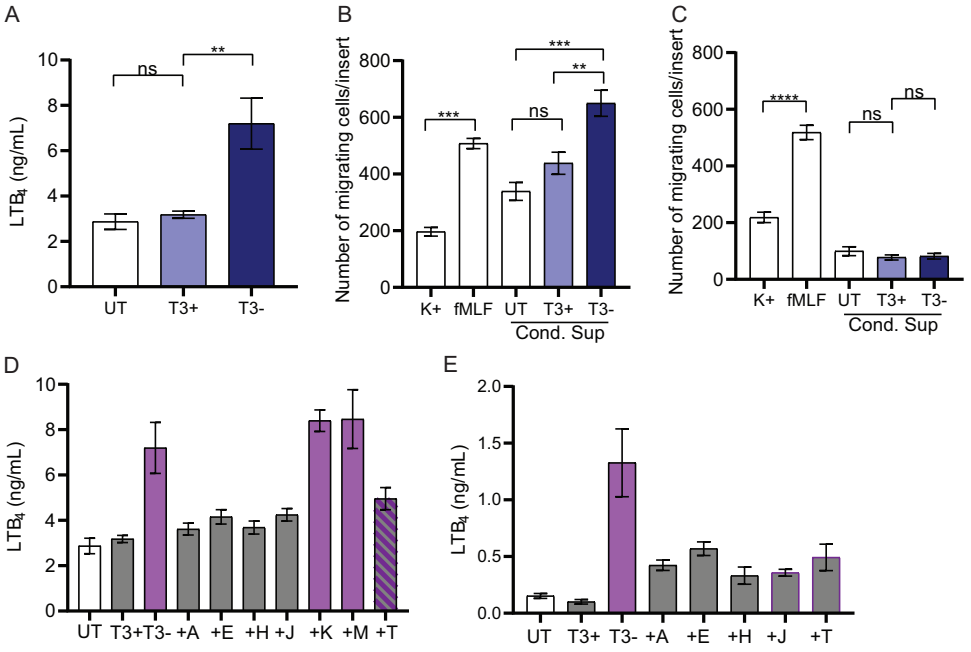

FIG 6 *Y. pestis* inhibits human neutrophil LTB_4 response. Human neutrophils (4×10^6) were infected with *Y. pestis* KIM1001 with or without the pCD1 plasmid encoding the T3SS ($T3^+$ or $T3^-$, respectively); MOI = 100. (A) Release of LTB_4 was measured after 30 min of infection in supernatant. (B and C) Chemotaxis of naive neutrophils in response to conditioned supernatant (B) without or (C) with pretreatment of the BLT1 inhibitor LY293111. (D) LTB_4 concentrations in the supernatant or (E) cell lysates of neutrophils infected with strains expressing only *ypkA* (+A), *yopE* (+E), *yopH* (+H), *yopJ* (+J), *yopK* (+K), *yopM* (+M), or *yopT* (+T). Mean ± SEM from 5 biologically independent experiments. One-way ANOVA with Dunnett's *post hoc* test. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. (D and E) Gray bars are significantly different than $T3^-$ ($P \leq 0.05$); purple bars are significantly different from $T3^-/T3^+$ ($P \leq 0.05$); hatched bars are not significantly different from $T3^-$ or $T3^-/T3^+$.

artificially disrupting the actin cytoskeleton. To test this hypothesis, human neutrophils were incubated with latrunculin A, a chemical inhibitor of actin polymerization, prior to infection with *Y. pestis*, and LTB_4 released into the supernatant was measured. As previously observed, significantly higher levels of LTB_4 were secreted by neutrophils treated with the vehicle and infected with *Y. pestis* KIM1001 $T3^{(-)}$ than by vehicle-treated neutrophils infected with *Y. pestis* KIM1001 (Fig. 7A; $P < 0.01$). However, treatment with latrunculin A resulted in loss of LTB_4 release in response to the strain lacking the T3SS, supporting that actin cytoskeleton disruption by Yop effectors can inhibit the LTB_4 response in neutrophils.

Disruption of MAPK signaling inhibits LTB_4 synthesis in response to *Y. pestis* infection. YopJ does not directly impact the host cell cytoskeleton, but it is a potent inhibitor of MAPK signaling (79–82). Since MAPK signaling has been shown to control LTB_4 synthesis in other models (79–86), we hypothesized that YopJ inhibition of LTB_4 synthesis is mediated by disruption of MAPK signaling. *In vitro* data indicate that YopJ can interact with multiple kinases in this pathway, including MAP3K (e.g., the TGF-β activating kinase [TAK1]) and MAP2K (e.g., mitogen-activated kinase kinase 6 [MEK6]) (72, 79, 86–88). Because TAK1 represents the earliest point in MAPK signaling targeted by YopJ, we tested whether treatment of neutrophils with a TAK1 chemical inhibitor was sufficient to inhibit LTB_4 synthesis in response to *Y. pestis* KIM1001 $T3^{(-)}$. As expected, when cells were exposed to the drug vehicle, we observed a significant increase in LTB_4 release by neutrophils infected with *Y. pestis* KIM1001 $T3^{(-)}$ compared to that by neutrophils infected with *Y. pestis* KIM1001 (Fig. 7B; untreated, $P \leq 0.001$). However, addition of the TAK1-specific inhibitor (5Z)-7-oxozeaenol [(5Z)-7-oxo] inhibited this response by neutrophils, and no difference in LTB_4 concentration was observed in the supernatants of neutrophils infected with *Y. pestis* KIM1001 or *Y. pestis* KIM1001 $T3^{(-)}$ [Fig. 7B; (5Z)-7-oxo]. TAK1 signaling is upstream of the MAPKs ERK and

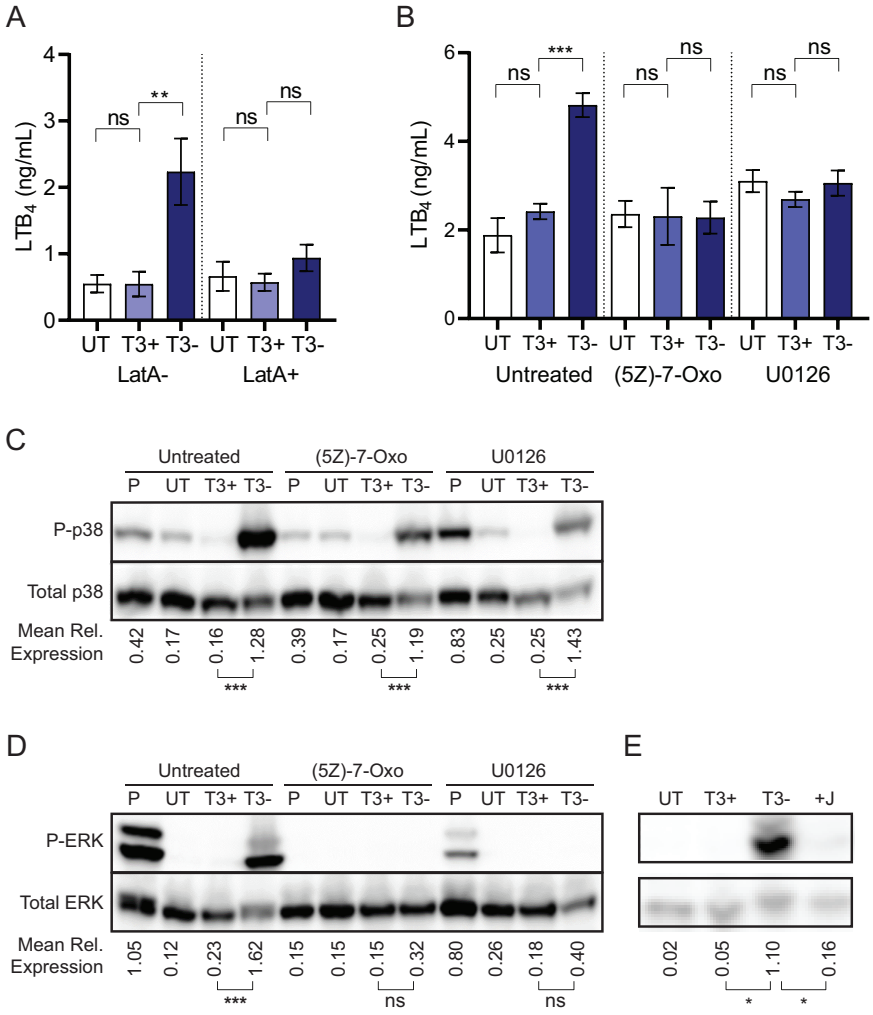

FIG 7 Inhibition of cytoskeletal rearrangement or MAPK signaling inhibits LTB_4 release. Human neutrophils (4×10^6 for LatA treatment or 8×10^6 for Western blots) were infected with *Y. pestis* KIM1001 with or without the pCD1 plasmid encoding the T3SS ($T3^+$ or $T3^-$, respectively); MOI = 100. (A) Concentration of LTB_4 in supernatant from infected neutrophils with indicated *Y. pestis* strains after pretreatment with vehicle control ($LatA^-$) or latrunculin A ($LatA^+$) prior to infection. (B) Concentration of LTB_4 in culture supernatants after infection with indicated *Y. pestis* strains after pretreatment with vehicle control (untreated), the TAK1 inhibitor (5Z)-7-oxozeaenol [(5Z)-7-Oxo], or the ERK inhibitor U0126. (C) Phosphorylation of p38 and (D) ERK during infection with indicated strains after pretreatment with vehicle control (untreated), the TAK1 inhibitor (5Z)-7-oxozeaenol [(5Z)-7-Oxo], or the ERK inhibitor U0126. (E) Phosphorylation of ERK during infection with indicated *Y. pestis* strains. $T3^+$, *Y. pestis* KIM1001; $T3^-$, *Y. pestis* KIM1001 $T3^{(-)}$; +J, KIM1001 expressing only *yopJ*; UT, uninfected. (A and B) Mean ± SEM from 5 biologically independent experiments. (C, D, and E) Mean relative expression calculated from 3 biologically independent Western blots. One-way ANOVA with Sidak's *post hoc* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

p38, but has not been shown to activate JNK in neutrophils (87). To determine which MAPK was impacted by inhibition of TAK1 signaling, cell lysates from infected neutrophils were harvested, and the levels of phosphorylated p38 and ERK were measured by Western blotting. Compared to untreated neutrophils, we observed no difference in the phosphorylation of p38 during *Y. pestis* infection in the presence of the TAK1 inhibitor [Fig. 7C; untreated versus (5Z)-7-oxo]. However, while phosphorylation of ERK was significantly increased in untreated cells during infection with *Y. pestis* KIM1001 $T3^{(-)}$, chemical inhibition of TAK1 signaling resulted in decreased ERK phosphorylation [Fig. 7D; (5Z)-7-oxo], indicating that TAK1-mediated activation of LTB_4 synthesis during *Y. pestis* KIM1001 $T3^{(-)}$ infection is through the ERK signaling pathway. To confirm that ERK signaling mediates LTB_4 production in response to *Y. pestis* KIM1001 $T3^{(-)}$, neu-

trophils were treated with the ERK-specific inhibitor U0126 prior to *Y. pestis* infection. Similarly to treatment with the TAK1 inhibitor, blocking ERK signaling with U0126 inhibited the release of LTB_4 in response to the *Y. pestis* KIM1001 $T3^{(-)}$ strain (Fig. 7B; U0126). Western blot analysis confirmed that U0126 specifically inhibited ERK phosphorylation and not p38 phosphorylation during *Y. pestis* KIM1001 $T3^{(-)}$ infection (Fig. 7C and D; U0126). Importantly, infection with *Y. pestis* KIM1001 expressing only YopJ recapitulated the inhibition of ERK phosphorylation observed during infection with *Y. pestis* expressing all of the Yop effectors (Fig. 7E), demonstrating that YopJ is sufficient to inhibit ERK signaling during *Y. pestis* infection. Together, these data indicate that inhibition of ERK signaling in neutrophils by YopJ is sufficient to inhibit LTB_4 synthesis during *Y. pestis* infection.

DISCUSSION

Through the T3SS and other virulence factors, *Y. pestis* is able to actively evade and inhibit the mammalian innate immune response, which allows the bacterium to colonize the host (75, 89, 90). Previous work has demonstrated targeting of resident and arriving neutrophils by *Y. pestis* for T3SS injection, which inhibits neutrophil antibacterial mechanisms that would otherwise result in bacterial killing (4, 34, 36, 39, 44, 63, 91–93). Specifically, *Y. pestis* has been shown to inhibit phagocytosis (9, 34), reactive oxygen species production (9, 34, 40), and production of cytokines (63) by neutrophils. Our study further expands the understanding of how *Y. pestis* impairs the inflammatory response of host neutrophils by inhibition of neutrophil degranulation and LTB_4 synthesis.

Work in the closely related species *Y. pseudotuberculosis* demonstrated that the T3SS actively inhibits neutrophil degranulation via the contributions of YopE and YopH (73). Our data, and a recent report from Eichelberger et al. (117), demonstrate that *Y. pestis* also utilizes these two effector proteins to inhibit neutrophil degranulation. However, by using a gain-of-function technique, we were also able to identify the contributions of YopJ and YpkA to the inhibition of specific and azurophilic granule exocytosis. Moreover, and importantly, we were able to show that multiple Yop effectors must act cooperatively to inhibit degranulation. The likely reasons YopJ and YpkA contributions were missed previously are because (i) four different protein combinations can inhibit degranulation of both specific and azurophilic granules, and (ii) while four proteins are involved, the bacterium requires either YopH or YopE (i.e., YopJ and YpkA cannot inhibit without YopE or YopH). Therefore, using a conventional loss-of-function deletion approach, a *yopE yopH* double mutant will have a phenotype, while any other double mutation combination will not, leading to the erroneous conclusion that YopE and YopH are redundant and sufficient to inhibit degranulation. These data also suggest the potential for hidden contributions of Yop effectors to other previously described phenotypes identified via loss-of-function mutational approaches. For example, while YopJ has been linked to inhibition of IL-8 by neutrophils, a *yopJ* mutant does not release as much IL-8 as a T3SS-deficient mutant, suggesting cooperative actions by other Yop effectors (63). Identification of other Yop effectors involved in inhibition could be performed using a similar gain-of-function approach to that described here.

Previous work has shown that YopE and YpkA target Rac signaling (54, 94, 95), YopH targets the focal adhesion complex (41), and YopJ targets the MAPK signaling pathway (59, 61, 79, 80, 82–84). All three of these host factors are key nodes in signaling pathways shown to be integral to regulating neutrophil granule release (96, 97). However, based on data from infections with single gain-of-function mutants, inhibition of one of these pathways by an individual Yop effector is not sufficient to inhibit degranulation. This suggests that individual signaling pathways may not be completely inhibited by the effector, or alternatively, that loss of signaling through one pathway can be compensated for in the neutrophil by signaling through the other pathways. While the latter hypothesis may be supported by our observation that YopE and YpkA, which both target the same node/pathway, are not able to inhibit degranulation, our data do not rule out the former, as some degree of signaling through this node may still

occur during coinfection with the YopE and YpkA strains. To overcome this hurdle, *Y. pestis* evolved to inhibit all three signaling pathways, with inhibition of at least two being sufficient to inhibit degranulation (an example of cellular process redundancy [98]). Importantly, the signaling pathways affected by these nodes are also important for other neutrophil antimicrobial mechanisms (16, 99–101). Therefore, by targeting these host factors, *Y. pestis* is able to simultaneously inhibit multiple arms of the neutrophil response to subvert the functions of host neutrophils.

While the contributions of YopH, YopE, YpkA, and YopJ to inhibition of degranulation were conserved for specific and azurophilic granules, coinfections with YopH and YopK only appeared to inhibit the release of azurophilic granules. Based on the described function of YopK, which is thought to primarily regulate the translocation of other Yop effectors into the host cell to evade inflammasome recognition (69), we were surprised that YopK enhanced inhibition during coinfection with YopH. While YopK is thought to act as a gatekeeper, regulating the translocation of the other effectors from inside the cell (102), it has not been shown to regulate the transport of effectors through the T3SS of other bacteria during coinfection of a cell (i.e., transcomplementation). While it is possible that during coinfection YopK is transregulating the levels of YopH translocated by other bacteria, it is not clear how this would enhance inhibition of degranulation of azurophilic granules or why this would not also impact specific granules. Alternatively, it is possible that YopK has other yet-to-be defined functions in the host cells, beyond its role as a gatekeeper, that contribute to this phenotype, and future studies with YopK should be open to this possibility.

While YopE, YopH, YpkA, and YopT disrupt the host actin cytoskeleton, translocation of YopT by itself resulted in a phenotype that differed from the other three—enhanced azurophilic granule exocytosis (Fig. 5B). Johnson et al. described Gem-interacting protein (GMIP), through RhoA GAP activity, controling actin remodeling around the secretory Rab27a-JCF1 positive subpopulation of azurophilic granules to facilitate exocytosis (103). Inhibition of actin polymerization by regulation of RhoA and ROCK activity releases the barrier that limits granule exocytosis (97). Therefore, inactivation of RhoA by YopT is likely responsible for this phenotype. However, since this phenotype is specific for YopT, this suggests that YopT targeting of RhoA is spatially or temporally distinct from that of the other Yop effectors, that YopE and YpkA do not target RhoA during neutrophil infection, or that different mechanisms of RhoA inactivation by individual Yop effectors (e.g., protease cleavage versus GAP activity) may result in different degrees/rates of inactivation. Importantly, the action of the other Yop effectors inhibits this enhanced degranulation response in the context of wild-type (WT) *Y. pestis* infection to protect the bacterium from release of azurophilic granules.

Individually, YpkA, YopE, YopH, YopJ, and YopT all appear to be sufficient to inhibit LTB_4 synthesis. Synthesis of LTB_4 requires activation and relocalization of the enzyme 5-lipooxygenase (5-LO) to a membrane such as the nucleus or endoplasmic reticulum or to recently described cytosolic structures called lipidosomes (23, 104, 105). In this active state, 5-LO rapidly converts arachidonic acid to LTA_4, which is followed by conversion to LTB_4 by LTA_4 hydrolase (23, 106). The mechanisms leading to 5-LO translocation are not well defined. Moreover, whether the rate-limiting step for initiation of LTB_4 synthesis is relocalization to membranes or bringing 5-LO in proximity to 5-LO activating protein (FLAP) is still uncertain. However, 5-LO is known to associate with two actin-interacting proteins, growth factor receptor-bound protein 2 (Grb2) and coactosin-like protein (CLP) (107). These interactions suggest that 5-LO translocation or interactions with FLAP require the actin cytoskeleton. This is further supported by our data, as four out of the five effectors that inhibit LTB_4 synthesis also disrupt the actin cytoskeleton. Moreover, treatment with the actin inhibitor latrunculin A also inhibited LTB_4 synthesis in response to *Y. pestis* $T3^{(-)}$. However, it is possible that individual effectors may inhibit the synthesis process at different steps, and identifying which steps are inhibited during *Y. pestis* infection is a future direction of our studies.

In addition to disruption of the host cytoskeleton, we have shown that *Y. pestis* is able to inhibit LTB_4 via YopJ disruption of ERK signaling. While YopJ inhibition of MAPK

signaling has been extensively studied in the context of macrophages (79, 80), to our knowledge, this is the first time YopJ inhibition of MAPK phosphorylation has been confirmed in neutrophils. Specifically, our data demonstrates that both ERK and p38 phosphorylation are inhibited during *Y. pestis* infection of neutrophils in a T3SS-dependent manner and that inhibition of TAK1-ERK signaling axis by YopJ is sufficient to inhibit LTB_4 synthesis (Fig. 7). In primary human neutrophils, TAK1 can differentially signal through ERK and p38, and phosphorylation of these MAPKs is dependent on the stimulus encountered by the neutrophils (87). For example, stimulation of neutrophils with lipopolysaccharide (LPS) results in TAK1-mediated phosphorylation of both ERK and p38, while stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) results in TAK1-mediated regulation of the MEK/ERK axis (90). Importantly, signaling via the TAK1-ERK pathway has also been shown to mediate LTB_4 synthesis by neutrophils in response to other chemoattractant factors (90), supporting our findings that targeting ERK signaling by YopJ contributes to inhibition of LTB_4 synthesis during *Y. pestis* infection. Importantly, MAPK signaling not only regulates LTB_4 synthesis in neutrophils but also induction of the respiratory burst, production of cytokines, and degranulation (87, 108–112). Therefore, targeting of MAPK signaling by YopJ and inhibition of TAK1-ERK-mediated signaling allows *Y. pestis* to disrupt many arms of the neutrophil response simultaneously.

In conclusion, *Y. pestis* is well adapted to surviving within the hostile host environment. Through this work, we found that neutrophils can only undergo granule exocytosis in response to *Y. pestis* infection when the T3SS is absent. In addition, the data presented here support previously described roles for YopE and YopH in inhibition of degranulation (73), while uncovering previously unidentified roles for YopJ and YpkA, which cooperatively work with YopE and YopH. Given these new data, we can also update the current model for inhibition of degranulation by *Y. pestis* to include the information that the bacterium needs to inhibit two of three signaling pathways to completely inhibit neutrophil degranulation. Moreover, *Y. pestis* also inhibits the synthesis of the potent chemoattractant LTB_4. Without LTB_4, neutrophil recruitment to the site of infection would be impaired. Moreover, as LTB_4 also stimulates macrophages toward enhanced phagosomal degradation of microorganisms (28) and promotes dendritic cell activation of T-cell responses (113–115), both of these important mechanisms to coordinate early antimicrobial responses by host innate immune cells are likely impaired during *Y. pestis* infection. Inhibition of neutrophil degranulation and LTB_4 production likely contributes to *Y. pestis* subverting the innate immune response and maintaining a noninflammatory host environment early during infection.

MATERIALS AND METHODS

Bacterial growth conditions. Bacterial strains used in these studies are listed in Table S1. Prior to infection, *Y. pestis* was cultured for 15 to 18 h at 26°C in Difco brain heart infusion (BHI) broth (BD Biosciences) with aeration. Cultures were diluted 1:10 in fresh BHI broth containing 20 mM $MgCl_2$ and 20 mM Na-oxalate and cultured at 37°C for 2 h with aeration to induce expression of the T3SS. Bacteria were centrifuged and resuspended in LPS-free Krebs-Ringer phosphate buffer (pH 7.2) containing 0.2% dextrose (Krebs) buffer for infection.

Human neutrophil isolation. Use of human neutrophils was approved by the University of Louisville's Institutional Review Board (IRB) guidelines (approval no. 96.0191). Neutrophils were isolated from peripheral blood of healthy, medication-free donors as described previously (10). Neutrophil isolations yielded ≥95% purity with ≥97% viability by Trypan blue exclusion staining and were used within 1 h of isolation.

Human neutrophil infection. Neutrophils (4×10^6; for Western blotting, 8×10^6 cells were used) were resuspended in Krebs buffer and, where indicated, incubated at room temperature (RT) for 30 min with 1 μM latrunculin A (catalog [cat.] no. 428021; Sigma), 20 μM U0126 (cat. no. 70970; Cayman), 50 nM LY293111 (cat. no. 10009768; Cayman), or 3 μM (5Z)-7-oxozeaenol (cat. no. 17459; Cayman). Neutrophils were infected at a multiplicity of infection (MOI) of 10 or 100 and incubated for 30 min in a 37°C water bath with gentle agitation. Coinfections were performed at a final MOI of 100 (50 for each strain), and bacteria were mixed together prior to adding to the cells. For specific and azurophilic granule exocytosis, the increases in plasma membrane expression of CD66b and CD63, respectively, were measured by flow cytometry as previously described (16). For CD63 plasma membrane expression, human albumin (cat. no. 521302; Alpha Therapeutic Corporation) was added to the cells (final concentration, 250 μg/ml) and incubated for an additional 5 min prior to staining. To measure release of gelatinase granules, secretory vesicles, or LTB_4, separate samples were centrifuged, and cell-free supernatants were transferred to new

tubes containing Halt phosphatase and protease inhibitor cocktail (cat. no. 78442; ThermoFisher Scientific) and stored at −80°C. Intracellular LTB_4 was measured by lysing neutrophils in ultrapure H_2O with phosphatase and protease inhibitors.

Measurement of exocytosis by flow cytometry and enzyme-linked immunosorbent assay. Neutrophils were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD63 (cat. no. 215-040; Ancell) or FITC-labeled anti-CD66b (cat. no. 305104; BioLegend) as markers for azurophilic and specific granules, respectively. As antibody isotype controls, neutrophils were separately labeled with FITC-labeled anti-IgM (cat. no. 401108; BioLegend) or FITC-labeled anti-IgG_1 (cat. no. 400108; BioLegend) on ice for 45 min before washing with FTA buffer (BD Biosciences) plus 0.05% sodium azide and fixing with 1% paraformaldehyde (PFA). Mean cellular fluorescence intensity (MCF) was measured using a fluorescence-activated cell sorting (FACS) Aria flow cytometer (BD Biosciences) with isotype control values subtracted as previously described (73). Albumin (cat. no. ab108788; abcam), MMP-9/gelatinase (cat. no. EK0465; Boster), and LTB_4 (cat. no. 520111; Cayman) levels were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols.

Chemotaxis assay. Supernatants from infected neutrophils were filtered using a 0.2 μm syringe filter to generate conditioned supernatants. Naive neutrophils (1×10^6 cells/ml) were loaded into the upper chamber of a 24-well Transwell plate (Corning). The lower chambers were filled with Krebs buffer, 100 nM fMLF (Sigma), or the conditioned supernatants. After incubation for 30 min at 37°C, neutrophils that migrated from the upper chamber to the lower side of the Transwell membranes were fixed and stained with Hema 3 (ThermoFisher) and counted by microscopy as described previously (116).

Western blotting. After 30 min of infection, cell pellets were obtained by centrifugation (6,000 × *g* for 30 s). Pellets were lysed using ice-cold lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [vol/vol] Triton X-100, 0.5% [vol/vol] Nonidet P-40, 20 mM NaF, 20 mM $NaVO_3$, 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM diisopropylfluorophosphate [DFP], 21 μg/ml aprotinin, and 5 μg/ml leupeptin). Lysates were mixed with Laemmli loading buffer and boiled for 10 min prior to snap cooling. Lysates were run on a 10% SDS-PAGE gel and immunoblotted with antibodies to phospho-ERK1/2, total ERK1/2, phospho-p38 MAPK, or total p38 MAPK (Cell Signaling) diluted 1:2,000 in 10 ml of Tris-buffered saline plus 0.1% Tween 20 (TBST) plus 5% bovine serum albumin (BSA). The appropriate secondary antibodies were used at 1:50,000 (cat. no. A9169; Sigma-Aldrich; cat. no. 31430; ThermoFisher Scientific). SuperSignal West Femto maximum-sensitivity substrate (cat. no. 34095; ThermoFisher Scientific) was used to detect antigen-antibody binding. Densitometry was performed using ImageJ software to quantify bands, normalized using the total protein form.

Statistics. Degranulation and LTB_4 data are the mean of five biologically independent experiments. Phosphorylation data are the mean of three biologically independent experiments. For all, neutrophils were harvested from both male and female donors and infections were performed on different days. Where appropriate, one-way analysis of variance (ANOVA) with Dunnett's or Sidak's post-test, as indicated in individual figure legends, was used for statistical analysis and performed using Prism 8 (GraphPad).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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