UC Santa Cruz

UC Santa Cruz Previously Published Works

Title

Impact of Host Membrane Pore Formation by the Yersinia pseudotuberculosis Type III Secretion System on the Macrophage Innate Immune Response

Permalink

https://escholarship.org/uc/item/2pg672bs

Journal

Infection and Immunity, 81(3)

ISSN

0019-9567

Authors

Kwuan, Laura
Adams, Walter
Auerbuch, Victoria

Publication Date

2013-03-01

DOI

10.1128/iai.01014-12

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

IAI Journals.ASM.org

Impact of Host Membrane Pore Formation by the *Yersinia pseudotuberculosis* Type III Secretion System on the Macrophage Innate Immune Response

Laura Kwuan, Walter Adams, Victoria Auerbuch

Department of Microbiology and Environmental Toxicology, University of California—Santa Cruz, Santa Cruz, California, USA

Type III secretion systems (T3SSs) are used by Gram-negative pathogens to form pores in host membranes and deliver virulence-associated effector proteins inside host cells. In pathogenic *Yersinia*, the T3SS pore-forming proteins are YopB and YopD. Mammalian cells recognize the *Yersinia* T3SS, leading to a host response that includes secretion of the inflammatory cytokine interleukin-1β (IL-1β), Toll-like receptor (TLR)-independent expression of the stress-associated transcription factor Egr1 and the inflammatory cytokine tumor necrosis factor alpha (TNF-α), and host cell death. The known *Yersinia* T3SS effector proteins are dispensable for eliciting these responses, but YopB is essential. Three models describe how the *Yersinia* T3SS might trigger inflammation: (i) mammalian cells sense YopBD-mediated pore formation, (ii) innate immune stimuli gain access to the host cytoplasm through the YopBD pore, and/or (iii) the YopB-YopD translocon itself or its membrane insertion is proinflammatory. To test these models, we constructed a *Yersinia pseudotuberculosis* mutant expressing YopD devoid of its predicted transmembrane domain ($YopD_{\Delta TM}$) and lacking the T3SS cargo proteins YopHEMOJTN. This mutant formed pores in macrophages, but it could not mediate translocation of effector proteins inside host cells. Importantly, this mutant did not elicit rapid host cell death, IL-1β secretion, or TLR-independent Egr1 and TNF-α expression. These data suggest that YopBD-mediated translocation of unknown T3SS cargo leads to activation of host pathways influencing inflammation, cell death, and response to stress. As the $YopD_{\Delta TM}$ *Y. pseudotuberculosis* mutant formed somewhat smaller pores with delayed kinetics, an alternative model is that the wild-type YopB-YopD translocon is specifically sensed by host cells.

Mammalian innate immune cells, such as macrophages, recognize and respond to numerous microbial invaders through a series of membrane-associated and cytoplasmic receptors that sense molecules unique to the microbial world (1–3). Based on the localization of these receptors and on the nature of their ligands, innate immune cells can distinguish between pathogenic and nonpathogenic bacteria (4). Given the trillions of commensal bacteria associated with the human body, the ability to induce an inflammatory response to potentially harmful pathogens while preventing chronic inflammation to beneficial commensal bacteria is of utmost importance to human health.

One clinically important, widespread, and evolutionarily ancient bacterial structure used specifically by Gram-negative pathogens is the type III secretion system (T3SS) (5). The T3SSs of several bacteria, including pathogenic *Yersinia* and *Pseudomonas aeruginosa*, are recognized by macrophages (6–9). This recognition leads to activation of the protease caspase-1 and secretion of the proinflammatory cytokine interleukin-1β (IL-1β) (7–9) as well as Toll-like receptor (TLR)-independent induction of numerous host genes, such as the stress-associated transcription factor Egr1 and the inflammatory cytokine tumor necrosis factor alpha (TNF-α) (6, 10). T3SS-stimulated secretion of IL-1β has been suggested to be important for neutrophil recruitment and protection against *P. aeruginosa* in a mouse lung infection model (11). This suggests that recognition of the T3SS by macrophages is an important component in defense against T3SS-expressing pathogens.

The T3SS resembles a molecular syringe composed of a basal structure that spans the bacterial inner and outer membranes and a needle-like structure that, in *Yersinia*, protrudes about 60 nm from the bacterial surface (12). At the tip of the *Yersinia* T3SS needle is LcrV, which coordinates the pore-forming proteins YopB and YopD in such a way that a pore is formed in the target host cell membrane. The LcrV-YopD-YopB translocon is thought to form a conduit that enables translocation of bacterial effector proteins called Yops directly inside the host cell cytoplasm, although this has never been visualized experimentally. The *Yersinia* T3SS targets cells of the innate immune system, such as macrophages (13–15). Once injected inside innate immune cells, the T3SS effector proteins interfere with uptake and killing of *Yersinia* and dampen some inflammatory responses (16). T3SS function is tightly regulated in *Yersinia*, such that host cell contact is required for robust translation and secretion of Yops. YopN is a T3SS regulatory protein that is secreted through the T3SS needle and serves to prevent premature secretion of effector proteins (17). In addition, the pore-forming protein YopD participates in a complex with the regulatory protein LcrQ, which inhibits translation of effector

Received 21 September 2012 Returned for modification 11 October 2012
Accepted 24 December 2012
Published ahead of print 7 January 2013
Editor: J. B. Bliska
Address correspondence to Victoria Auerbuch, vastone@ucsc.edu.
L. K. and W. A. contributed equally to this article.
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/IAI.01014-12

TABLE 1 *Y. pseudotuberculosis* strains used in this study

Strain	Background	Mutation(s)	Reference
Wild type	IP2666	Naturally lacks full-length YopT	30
Δ*yscNU* mutant	IP2666	Δ*yscNU*	31
Δyop6 mutant	IP2666	Δ*yopHEMOJ*	6
Δyop6 Δ*yopN* mutant	IP2666	Δ*yopHEMOJN*	6
Δyop6 Δ*yopN* Δ*yopB* mutant	IP2666	Δ*yopHEMOJNB*	This work
Δyop6 Δ*yopN* Δ*yopD* mutant	IP2666	Δ*yopHEMOJND*	This work
Δyop6 Δ*yopN* $yopD_{\Delta TM}$ mutant	IP2666	Δ*yopHEMOJN* $yopD_{\Delta 128–149}$	This work
Δyop6 Δ*yopN*(pYopT) mutant	IP2666	Δ*yopHEMOJN*(pPHYopT)	This work
Δyop6 Δ*yopN* $yopD_{\Delta TM}$(pYopT) mutant	IP2666	Δ*yopHEMOJN* $yopD_{\Delta 128–149}$(pPHYopT)	This work
Δyop6 Δ*yopK* mutant	IP2666	Δ*yopHEMOJK*	This work
Wild-type with pYopM-Bla	IP2666	pMM83::*yopM-bla* fusion	This work
Δ*yopB*(pYopM-Bla) mutant	IP2666	Δ*yopB*(pMM83::*yopM-bla*) fusion	This work
Δyop6 Δ*yopN*(pYopM-Bla) mutant	IP2666	Δ*yopHEMOJTN*(pMM83::*yopM-bla*) fusion	This work
Δyop6 Δ*yopN* Δ*yopB*(pYopM-Bla) mutant	IP2666	Δ*yopHEMOJTNB*(pMM83::*yopM-bla*) fusion	This work
Δyop6 Δ*yopN* Δ*yopD*(pYopM-Bla) mutant	IP2666	Δ*yopHEMOJTND*(pMM83::*yopM-bla*) fusion	This work
Δyop6 Δ*yopN* $yopD_{\Delta TM}$(pYopM-Bla) mutant	IP2666	Δ*yopHEMOJTN* $YopD_{\Delta TM}$(pMM83::*yopM-bla*) fusion	This work

protein mRNA prior to host cell contact (18–20). Once host cell contact is made, derepression of YopN secretory control allows secretion of LcrQ and translation of effector protein mRNA. Through these mechanisms and others, *Yersinia* prevents robust expression and secretion of effector proteins until they are required.

In addition to translocation of effector proteins, the T3SS of several pathogens has been shown or suggested to deliver flagellin and a T3SS structural protein called the inner rod protein inside host cells (21, 22). Flagellin and inner rod proteins are directly recognized by cytoplasmic innate immune receptors called Naips, which together with Nlcr4/Ipaf activate caspase-1, stimulating secretion of IL-1β in addition to other responses (23, 24). However, another cytoplasmic innate immune receptor, called Nlrp3/Nalp3, has been implicated in stimulating activation of caspase-1 and IL-1β secretion in response to the T3SS (25). Nlrp3 responds to a wide variety of stimuli, including bacterial pore-forming toxins, possibly through detection of potassium efflux, although the mechanism remains unclear (3, 25). The dominant pathway contributing to caspase-1 activation in response to the *Yersinia* T3SS has been shown to be the Nlrp3 pathway (26). As mice lacking caspase-1 are more susceptible to *Yersinia pseudotuberculosis* infection (26), elucidation of how the *Yersinia* T3SS triggers caspase-1 activation is critical for our overall understanding of *Yersinia* virulence. Furthermore, it remains unclear how the *Yersinia* T3SS triggers TLR-independent expression of host genes, such as Egr1 (6), as these responses are thought to be independent of caspase-1.

Six known *Yersinia* T3SS translocated proteins, YopHEMOJT, are dispensable for triggering IL-1β secretion and TLR-independent Egr1 and TNF-α expression (6). However, the pore-forming protein YopB is necessary for host cell innate immune recognition of the T3SS (T3SS recognition) (6–8, 27, 28). This requirement indicates that a YopB-mediated event, YopB membrane insertion, pore formation, and/or translocation of unknown T3SS cargo, results in T3SS recognition. Specifically, membrane disruption or potassium efflux through the T3SS pore has been suggested to underlie T3SS-triggered host cell signaling (6, 26, 29). To understand the critical requirements for T3SS recognition, we sought to determine whether T3SS-mediated pore formation alone was sufficient to trigger host responses in macrophages. We identified a *Y. pseudotuberculosis* mutant that lacks YopHEMOJTN and expresses an allele of YopD devoid of its transmembrane domain ($yopD_{\Delta TM}$). This Δyop6 Δ*yopN* $yopD_{\Delta TM}$ mutant secreted YopB and formed 1.3- to 3.8-nm pores in macrophages, but it was incapable of translocating T3SS cargo inside host cells. Importantly, the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain did not induce IL-1β secretion, rapid host cell death, or TLR-independent expression of Egr1 and TNF-α. These data show that a translocation-incompetent YopBD pore is insufficent to stimulate a significant immune response. These findings suggest that either a wild-type YopB-YopD translocon or translocation of molecules distinct from YopHEMOJTN activates several macrophage signaling pathways that control inflammatory cytokine production, response to stress, and host cell survival.

MATERIALS AND METHODS

Bacterial growth conditions. *Y. pseudotuberculosis* was grown in 2× YT (yeast extract-tryptone) at 26°C with shaking overnight. The cultures were back-diluted into low-calcium medium (2× YT plus 20 mM sodium oxalate and 20 mM $MgCl_2$) to an optical density at 600 nm (OD_{600}) of 0.2 and grown for 1.5 h at 26°C with shaking followed by 1.5 h at 37°C with shaking to induce Yop synthesis as previously described (6).

Bacterial mutants. The bacterial strains used in this study are listed in Table 1. The *Y. pseudotuberculosis* strains generated for this study were constructed by splicing by overlap extension PCR. Primers were designed using MacVector and Primer 3 software (http://fokker.wi.mit.edu/primer3/input.htm). Amplified PCR fragments, encoding ~200 to 400 bp of homology on either side of the intended mutation, were cloned into a SalI- and NotI-digested pSR47s suicide plasmid (λ*pir*-dependent replicon, Kan^r, with a *sacB* gene conferring sucrose sensitivity) (32, 33). Recombinant plasmids were transformed into *Escherichia coli* S17-1λ*pir* competent cells and later introduced into *Y. pseudotuberculosis* IP2666 via conjugation. The resulting Kan^r and Irgasan-resistant integrants were grown in the absence of antibiotic selection and plated on sucrose-containing media to identify clones that had lost *sacB* (and by inference, the linked plasmid DNA). Kan^s, sucrose-resistant, Congo red-positive colonies were screened by PCR and subsequently sequenced to confirm the presence of the intended mutation. The $yopD_{\Delta TM}$ mutation was constructed using the internal primers described by Olsson et al. (34) along with the external primers 5′-CGGTCACTAGTGCCAGAATTGATC-3′ and 5′-TTGGCGTCGAC

GTCGGTCATTCTG-3′. The full-length *yopD* deletion mutation (leaving only the first three and last three amino acids of YopD) was constructed using the primers described in reference 35. The Δ*yopK* mutation was introduced into *Y. pseudotuberculosis* using a pSR47s-derived suicide plasmid obtained from Molly Bergman (unpublished data). Melanie Marketon kindly provided *E. coli* expressing YopM–β-lactamase (Bla) reporter plasmids containing a chloramphenicol resistance (Cm^r) cassette (36). YopM-Bla reporter plasmids were isolated and electroporated into *Y. pseudotuberculosis*. Single colonies were selected by plating on chloramphenicol and Congo red plates.

Primary cells and cell lines. All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (37) and were approved by the UC Santa Cruz Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from The Jackson Laboratory. Primary bone marrow-derived macrophages (BMDMs) were prepared as previously described (6). Immortalized C57BL/6 and $MyD88^{-/-}$ $Trif^{-/-}$ BMDMs were obtained from Douglas Golenbock and were grown routinely in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO_2. Immortalized BMDMs were used for most assays to minimize animal use. However, immortalized C57BL/6 BMDMs secreted minimal IL-1β following *Y. pseudotuberculosis* infection (with or without prior TLR stimulation), so primary BMDMs were used for measurement of the release of IL-1β upon *Y. pseudotuberculosis* infection. However, the overall trends were similar between primary and immortalized BMDMs (W. Adams and V. Auerbuch, unpublished data).

Hemolysis. Red blood cell (RBC) lysis induced by *Y. pseudotuberculosis* was measured as previously described, with some alterations (38). *Y. pseudotuberculosis* low-calcium-medium cultures (see "Bacterial growth conditions" above) were spun down and resuspended at 2×10^9/ml. Sheep red blood cells (sRBC; Innovative Research) were washed three times with ice-cold phosphate-buffered saline (PBS) and resuspended in low-calcium medium at 2×10^9/ml. Fifty microliters each of sRBC and bacterial suspension was added in triplicate to a 96-well round-bottom plate (BD Falcon), centrifuged at 4,000 rpm for 15 min at room temperature, and incubated at 37°C for 2 h. The supernatant was removed, and the remaining pellet was resuspended in 150 μl ice-cold PBS plus 10 μg/ml gentamicin or water. The resuspended pellets were incubated overnight at 26°C and centrifuged at 4,000 rpm for 5 min at 4°C. A total of 150 μl of the supernatant was transferred to a 96-well clear-bottom white plate (Corning), and absorbance was read at 545 nm on a Victor^3 plate reader (PerkinElmer). Data from three separate wells were averaged for each independent experiment.

BCECF release. BCECF [2′,7′-bis-(2-carboxyethyl)-5 (and 6)-carboxyfluorescein] release was performed as previously described, with some alterations (39). A total of 4×10^5 immortalized C57BL/6 BMDMs were plated in each well of a 24-well plate (BD Falcon) in 1 ml DMEM plus 10% FBS and incubated overnight. Twenty minutes prior to infection, BMDMs were washed twice with 1 ml PBS and incubated with 500 μl of Hanks' balanced salt solution (HBSS) plus 10 μM BCECF-AM (BCECF acetoxymethyl ester derivative) (Invitrogen) for 30 min at 37°C with 5% CO_2. Cells were washed twice in warmed phenol red-free RPMI. The BCECF-loaded BMDMs were infected at a multiplicity of infection (MOI) of 100, and the infected monolayer was centrifuged at 400 × *g* at 4°C to initiate contact. The cells were then incubated at 37°C with 5% CO_2 for 1 or 2 h. Alternatively, 0.09% Triton X-100 was added to the cells 45 min prior to the completion of the experiment to achieve 100% BCECF release. The cells were centrifuged for 4 min at 250 × *g*, 140 μl of cell culture supernatant was transferred in triplicate into a 96-well clear-bottom white plate (Corning), and BCECF fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm with a Victor^3 plate reader (PerkinElmer). The average of three separate BCECF fluorescence measurements was averaged for each independent experiment. The percentage of BCECF release was calculated as [(sample − uninfected)/(Triton X-100 − uninfected)] × 100. Osmoprotectants, polyethylene glycol (PEG) 6000 (Spectrum), PEG 3350 (Calbiochem), sucrose (Fischer), or raffinose (Alfa Aesar), were resuspended in warm phenol red-free RPMI and added at a 30 mM final concentration in a total volume of 0.5 ml (40).

EtBr entry assay. A total of 2×10^4 immortalized C57BL/6 BMDMs were plated in each well of a 96-well clear-bottom black plate (Corning) in 100 μl DMEM plus 10% FBS and incubated overnight. The cells were infected in triplicate at an MOI of 25 or 100 and centrifuged for 5 min at 750 × *g* at 4°C to initiate contact. The cells were then incubated at 37°C with 5% CO_2 for 2 h. At the end of the incubation period, the medium was aspirated and replaced with 30 μl of PBS containing 25 μg/ml ethidium bromide (EtBr) and 12.3 μg/ml Hoechst dye (Fischer). The cell monolayer was visualized using an ImageXpress^{MICRO} automated microscope and MetaXpress analysis software (Molecular Devices). The percentage of EtBr-positive cells was calculated by dividing the number of EtBr-stained cells by the number of Hoechst-stained cells. Data from three separate wells were averaged for each independent experiment.

Cell rounding assay. A total of 2×10^5 immortalized $MyD88^{-/-}$ $Trif^{-/-}$ BMDMs were plated in each well of a 24-well plate in 1 ml DMEM plus 10% FBS and incubated overnight. $MyD88^{-/-}$ $Trif^{-/-}$ BMDMs were chosen because of their flatter morphology (unpublished observations). The $MyD88^{-/-}$ $Trif^{-/-}$ BMDMs were infected with *Y. pseudotuberculosis* expressing YopT at an MOI of 15. Cell rounding was monitored using an Olympus CKX41 microscope by two or three different researchers who were blinded to the identity of each sample.

YopM translocation assay. A total of 2×10^4 CHO-K1 cells were plated in each well of a 96-well plate in 100 μl of F-12K medium plus 10% FBS and incubated overnight. CHO-K1 cells were infected with *Y. pseudotuberculosis* β-lactamase reporter strains for 2 h at an MOI of 30 and incubated at 37°C with 5% CO_2. Thirty to 45 min prior to the end of the infection, 20 μl of 6× loading solution containing CCF2-AM (Invitrogen) was added to each well, and the plate was covered in foil and incubated at 30°C with 5% CO_2. At the end of infection, the medium was aspirated and 5 μM DRAQ5 in PBS was added to each well. The monolayers were incubated at room temperature for 5 min, washed once with PBS, and visualized using an ImageXpress^{MICRO} automated microscope and MetaXpress analysis software (Molecular Devices). The number of YopM-Bla-positive cells was calculated by dividing the number of blue (CCF2-cleaved) cells by the number of green (total $CCF2^+$) cells. Data from two separate wells were averaged for each independent experiment.

Type III secretion assay. Visualization of T3SS cargo secreted in broth culture was performed as previously described (6). *Y. pseudotuberculosis* low-calcium-medium cultures were grown for 1.5 h at 26°C and switched to 37°C for another 2 h. Cultures were spun down at 13,200 rpm for 10 min at room temperature. Supernatants were transferred to a new Eppendorf tube. A 10% final concentration of trichloroacetic acid was added, and the mixture was vortexed vigorously. Samples were incubated on ice for 20 min and then spun down at 13,200 rpm for 15 min at 4°C. The pellet was resuspended in final sample buffer (FSB) plus 20% dithiothreitol (DTT). Samples were boiled for 5 min prior to running on a 12.5% SDS-PAGE gel. Sample loading was normalized for number of bacteria per sample.

LDH release assay. A total of 1.25×10^6 immortalized C57BL/6 BMDMs were plated in each well of a 6-well plate (BD Falcon) in 2 ml DMEM plus 10% FBS and incubated overnight. The cells were infected at an MOI of 15. Ninety microliters of supernatant was transferred to an Eppendorf tube every hour for 4 h. After the last time point, the cells were freeze-thawed to achieve full lactate dehydrogenase (LDH) release. Supernatants were centrifuged for 1 min at 13,000 rpm, and 50 μl was transferred to a 96-well clear-bottom white plate (Corning). LDH release into the supernatant was measured using the CytoTox 96 nonradioactive cytotoxicity assay according to the manufacturer's instructions (Promega). LDH release as a result of freeze-thaw was set at 100% for each sample.

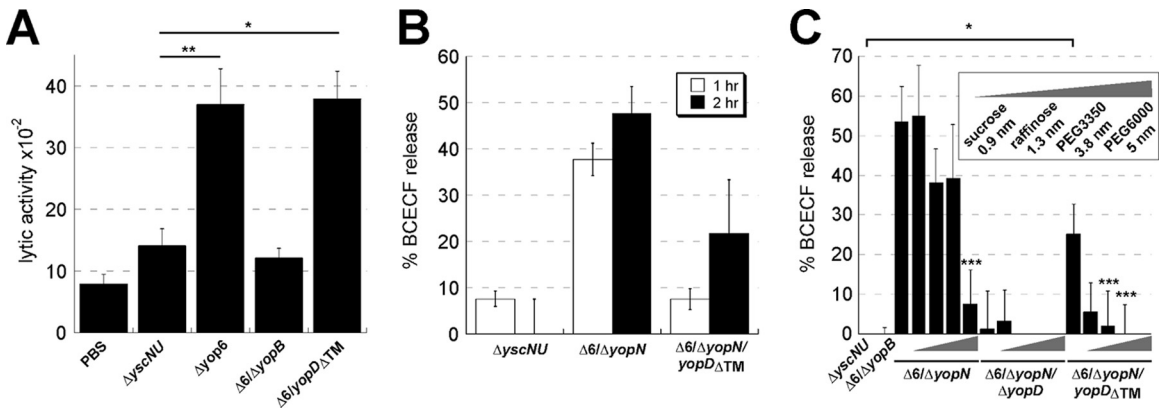

FIG 1 Analysis of *Y. pseudotuberculosis*-induced pore formation on red blood cells and macrophages by monitoring release of hemoglobin or BCECF. (A) Red blood cells were incubated with *Y. pseudotuberculosis*, and the amount of hemoglobin released as a result of pore formation was measured. The averages ± standard errors of the means (SEM) of the raw absorbance readings from three to nine independent experiments are shown. *, $P \leq 0.05$, as determined by one-way ANOVA followed by Bonferroni's *post hoc* test (comparing Δ*yscNU*, Δyop6 [Δ6], and Δyop6 *yopD*$_{\Delta TM}$ strains). (B) BCECF-loaded immortalized C57BL/6 BMDMs were infected with *Y. pseudotuberculosis* at an MOI of 100. The amount of BCECF fluorescence released into the supernatant as a result of pore formation was measured 1 or 2 h postinoculation. The averages ± SEM from two independent experiments are shown. (C) The experiment in panel B was repeated in the presence of 30 mM osmoprotectants of different diameters (see inset). *, $P \leq 0.05$, **, $P \leq 0.005$, and ***, $P \leq 0.0005$, as determined by one-way ANOVA followed by Bonferroni's *post hoc* test, where each indicated group was compared to the appropriate negative and positive controls (Δ*yscNU* and Δyop6 Δ*yopN* or Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strains).

IL-1β protein detection. A total of 5×10^5 primary C57BL/6 BMDMs were plated in each well of a 24-well plate in 1 ml DMEM plus 10% FBS and incubated overnight. The cells were infected at an MOI of 20, and 900 μl of the supernatant was removed at 4 h, as previously described (6). IL-1β protein levels in the supernatant were measured using a mouse Platinum IL-1β enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (eBioscience).

qPCR. A total of 1.25×10^6 immortalized MyD88$^{-/-}$ Trif$^{-/-}$ BMDMs were plated in each well of a 6-well plate in 2 ml DMEM plus 10% FBS and incubated overnight. The cells were infected with *Y. pseudotuberculosis* at an MOI of 15, the supernatant was filtered at 30 min through a 0.22-μm-pore filter (Millipore) to remove nonattached and noninternalized bacteria, and total RNA was harvested at 2 h as previously described (6). Transcript levels of TNF-α, Egr1, and 18S rRNA were measured as previously described (6). Data from three separate quantitative PCRs (qPCRs) was averaged for each independent experiment.

Statistical analysis. Statistical significance of differences between groups was calculated using one-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test (95% confidence level), using KaleidaGraph (Synergy Software).

RESULTS

The *Y. pseudotuberculosis* Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strain forms pores in macrophages. In order to determine the effects of pore formation on the host response, we sought to identify a *Y. pseudotuberculosis* T3SS mutant that forms pores in host cell membranes but cannot translocate T3SS cargo. Several *Y. pseudotuberculosis* mutants were previously described to make pores in red blood cells (RBCs) but were incapable of translocating T3SS effector proteins inside nucleated cells (34). One mutant (Δ*yopD*$_{TM}$) had a small internal in-frame deletion within YopD spanning amino acids 128 to 149, which encompasses the predicted YopD transmembrane (TM) domain and is involved in binding to the YopBD chaperone protein LcrH/SycD (34). We constructed an identical YopD$_{\Delta TM}$ mutation in the *Y. pseudotuberculosis* IP2666 strain lacking the six T3SS effector proteins YopHEMOJT (Δyop6), as we have previously ruled out a role for YopHEMOJT in triggering IL-1β secretion and TLR-independent Egr1 and TNF-α expression (6). To verify that this mutant and several control strains can insert pores in RBCs as previously reported, we monitored hemoglobin release from sheep RBCs following infection with *Y. pseudotuberculosis* (see Materials and Methods) (Fig. 1A). Consistent with Olsson et al. (34), the *Y. pseudotuberculosis* Δyop6 *yopD*$_{\Delta TM}$ strain induced significant hemoglobin release, suggesting robust pore formation in RBCs.

It was critical to determine the ability of this mutant to form pores in macrophages, not just RBCs, as macrophages have been shown to recognize and respond to the *Yersinia* T3SS and are a major target of the *Yersinia* T3SS *in vivo* (13–15). To test the ability of the *Y. pseudotuberculosis* Δyop6 *yopD*$_{\Delta TM}$ strain to form pores in macrophages, we analyzed the ability of this strain to allow the release of the small fluorescent dye BCECF from immortalized, murine bone-marrow-derived macrophages (BMDMs) 2 h postinoculation. Neither the Δyop6 *yopD*$_{\Delta TM}$ mutant nor the Δyop6 strain (which expresses wild-type YopD) could trigger release of BCECF even at a high multiplicity of infection (data not shown). These data highlight important differences in the sensitivity of pore formation assays and emphasize that requirements for pore formation in RBCs and nucleated cells may differ.

As YopN-deficient *Y. enterocolitica* was previously shown to induce significantly greater BCECF release than YopN-positive strains, we deleted YopN from the Δyop6 and Δyop6 *yopD*$_{\Delta TM}$ mutants (26). Growth of the Δyop6 Δ*yopN* and Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strains was identical under the culture conditions used in the BCECF release assay (data not shown). The *Y. pseudotuberculosis* Δyop6 Δ*yopN* strain induced release of 40 to 50% of total intracellular BCECF by 1 or 2 h postinoculation (Fig. 1B). The *Y. pseudotuberculosis* Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strain showed slower BCECF release kinetics but did trigger release of 20 to 25% of total BCECF 2 h postinoculation (Fig. 1B, $P = 0.09$, $n = 2$; C, $P < 0.05$, $n = 5$). These data suggest that the *Y. pseudotuberculosis* Δyop6 Δ*yopN yopD*$_{\Delta TM}$ mutant is capable of forming pores in macrophages.

In order to confirm this finding, we used a distinct pore for-

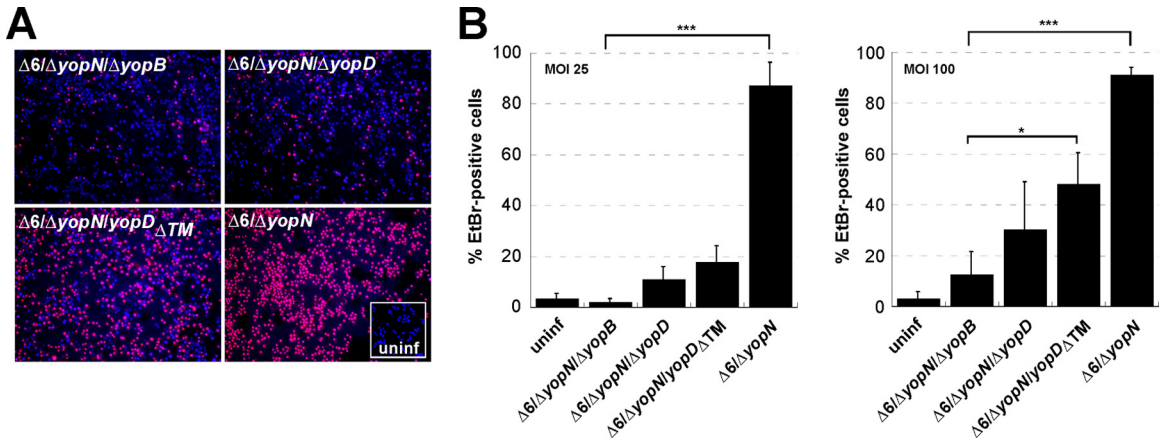

FIG 2 Analysis of *Y. pseudotuberculosis*-induced pore formation on macrophages by monitoring entry of ethidium bromide. (A and B) Entry of ethidium bromide (EtBr) inside *Yersinia*-infected immortalized C57BL/6 BMDMs was monitored 2 h postinoculation using fluorescence microscopy. (B) The number of EtBr-positive cells out of the total Hoechst-positive cells was quantified 2 h postinoculation. The averages ± SEM from three to five independent experiments are shown. *, $P \leq 0.05$, and ***, $P \leq 0.0005$, as determined by one-way ANOVA followed by Bonferroni's *post hoc* test, where each indicated group was compared to the appropriate negative and positive controls (Δyop6 Δ*yopN* Δ*yopB* and Δyop6 Δ*yopN* strains). uninf, uninfected.

mation assay that measures the entry of ethidium bromide (EtBr) inside infected BMDMs by fluorescence microscopy (41). Two hours postinoculation, the amount of EtBr entry inside BMDMs during infection with the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ mutant was significantly greater than during infection with the *yopB*-minus strain and ranged from 24 to 78% of total cells scoring positive (Fig. 2B). Taken together, these data demonstrate that the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain forms pores in macrophages.

The *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain expresses an allele of YopD that is predicted to interact with the host membrane very differently from wild-type YopD, yet this strain can form pores in RBCs and macrophages. Likewise, other substantial modifications to the translocon complex of T3SSs have resulted in mutants that retain the capacity for membrane disruption (42). For example, the *Pseudomonas aeruginosa* YopB homologue PopB has been shown to disrupt membranes in the absence of the YopD homologue PopD (43). We asked whether a complete YopD deletion mutant would also form pores in mammalian cells. We constructed a *Y. pseudotuberculosis* mutant carrying a full, in-frame deletion of YopD (Δyop6 Δ*yopN* Δ*yopD*). While the average number of EtBr-stained cells during Δyop6 Δ*yopN* Δ*yopD* strain infection was greater than in the *yopB*-minus infection, this difference was not statistically significant by one-way ANOVA (see Materials and Methods) and was only significant at an MOI of 25 by the Student *t* test (Fig. 2B, $P = 0.05$). Collectively, these data suggest that a YopB-$YopD_{\Delta TM}$ translocon forms pores on macrophages, but pore formation is either nonexistent or just below the level of detection in the complete absence of YopD.

The *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain forms 1.3- to 3.8-nm pores in macrophages. In order to further compare the pores formed by the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain to those formed when wild-type YopD is expressed, we measured pore size using an osmoprotection assay. We infected BMDMs with *Y. pseudotuberculosis* in the presence of osmoprotectants of different sizes and measured the amount of BCECF release 2 h postinoculation. Only osmoprotectants larger than the diameter of the T3SS pore can prevent osmotic lysis. By using different sizes of osmoprotectants, the approximate diameter of the pore can be determined (44). The size of the *Y. pseudotuberculosis* Δyop6 Δ*yopN* pore was 3.8 to 5 nm, as only PEG 6000 was able to significantly block BCECF release (Fig. 1C). In contrast, the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ pore was 1.3 to 3.8 nm, as both PEG 3350 and PEG 6000 significantly blocked BCECF release (Fig. 1C). These data suggest that a YopB-$YopD_{\Delta TM}$ translocon forms smaller pores than a wild-type translocon. However, the pores formed by the Δyop6 Δ*yopN* $yopD_{\Delta TM}$ mutant are still well within the range previously reported for T3SSs (12, 42).

The *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain cannot translocate ectopically expressed T3SS effector proteins. In order to determine whether the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain is capable of translocating T3SS cargo inside host cells, we measured entry of two different effector proteins inside mammalian cells—YopT and YopM. Translocation of plasmid-encoded YopT inside BMDMs was analyzed by measuring cell rounding, a downstream consequence of the Rho GTPase-disrupting activity of YopT (45). While *Y. pseudotuberculosis* encoding wild-type YopB and YopD caused 100% of the BMDMs to round by 11 h postinoculation, the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain did not cause cell rounding over the background Δyop6 Δ*yopN yopB* level (40%) (data not shown).

To confirm that the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain cannot translocate T3SS cargo inside host cells, we measured translocation of a plasmid-encoded YopM–β-lactamase (YopM-Bla) reporter protein inside CHO cells using the fluorescent β-lactamase substrate CCF2 (36). While the *Y. pseudotuberculosis* Δyop6 Δ*yopN* strain translocated YopM-Bla similarly to wild-type *Y. pseudotuberculosis*, strains lacking YopB or YopD were incapable of any YopM-Bla translocation (Fig. 3A and B). Importantly, the *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain was also deficient in YopM-Bla translocation (Fig. 3A and B), although it was capable of secreting YopM-Bla, YopD, and YopB into the culture supernatant upon growth under T3SS-inducing conditions (Fig. 3C). These data show that while the *Y.*

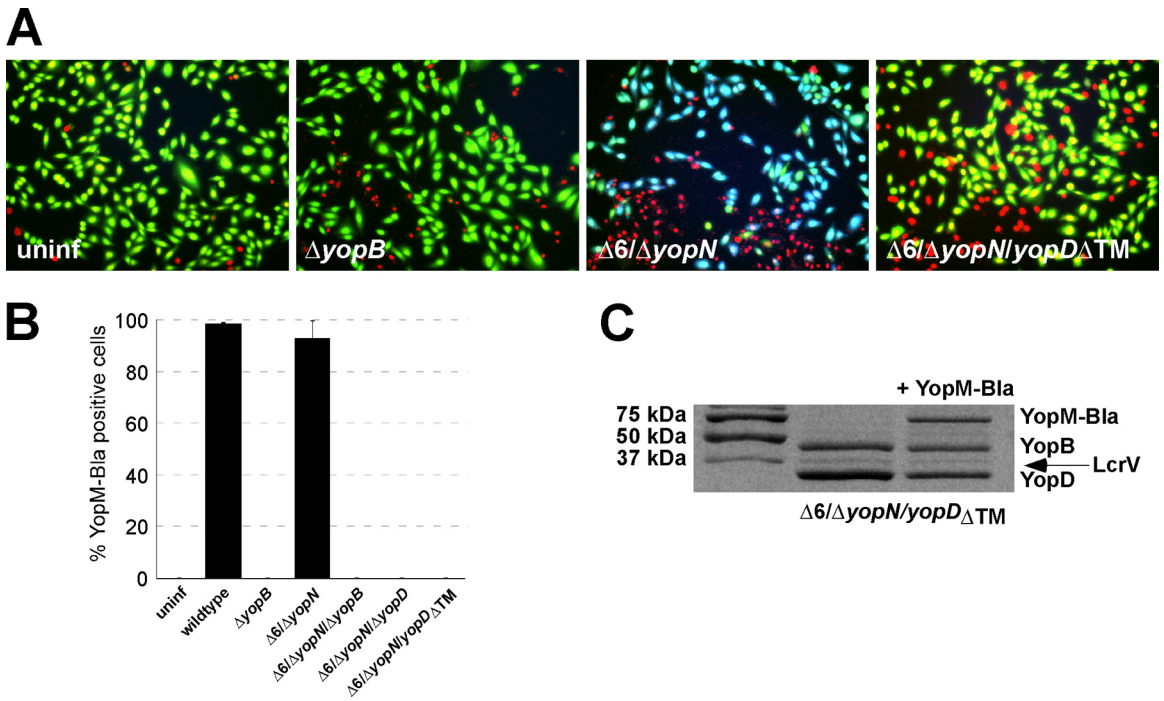

FIG 3 Examination of YopM–β-lactamase translocation inside CHO cells. (A and B) CHO cells were loaded with the fluorescent β-lactamase substrate CCF2 and infected with *Y. pseudotuberculosis* expressing a YopM–β-lactamase (YopM-Bla) reporter construct for 2 h. Green (uncleaved CCF2) and blue (cleaved CCF2) fluorescence was measured and used to determine the percentage of CHO cells that had been injected with YopM-Bla: % YopM-Bla-positive cells = blue CCF2-cleaved cells/green total $CCF2^+$ cells × 100%. (B) Results are representative of two independent experiments. (C) *Y. pseudotuberculosis* was grown in low-calcium medium at 37°C to induce type III secretion in the absence of host cells. Proteins in the bacterial culture supernatant were precipitated and visualized on a polyacrylamide gel using Coomassie blue.

pseudotuberculosis Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strain can secrete T3SS cargo, it fails to translocate T3SS cargo inside mammalian cells.

The *Y. pseudotuberculosis* Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strain does not trigger rapid cell death, IL-1β secretion, or TLR-independent Egr1 and TNF-α expression in macrophages. Mammalian cells infected with *Y. pseudotuberculosis* Δ*yopEHJ* or *Yersinia enterocolitica* Δ*yopHEMOPN* strains die after several hours of infection, releasing the large cytoplasmic protein lactate dehydrogenase (27, 39). As BCECF release precedes LDH release, LDH release has been previously ascribed to osmolysis resulting from YopBD-mediated pore formation (39). In agreement with these previous publications, the *Y. pseudotuberculosis* Δyop6 Δ*yopN* strain induced significant LDH release from BMDMs by 2 h postinoculation (Fig. 4A). However, the *Y. pseudotuberculosis* Δyop6 Δ*yopN yopD*$_{\Delta TM}$ and Δyop6 Δ*yopN yopD* strains did not induce LDH release until 4 h postinoculation, and even at this late time point, the amount of LDH released was lower than that in the Δyop6 Δ*yopN* infection. These data show that *Yersinia* expressing a YopB-YopD translocon competent for pore formation but not translocation cannot induce rapid cell death (i.e., prior to 4 h postinoculation). We propose that the rapid cell death induced by the Δyop6 Δ*yopN* strain may be triggered by translocation of unknown innate immune stimuli inside host cells.

The T3SSs of a number of pathogens, including *Yersinia*, have been shown to induce activation of the inflammatory protease caspase-1, leading to maturation and secretion of the inflammatory cytokine IL-1β (25). To test whether T3SS-mediated pore formation was capable of triggering IL-1β secretion, we infected BMDMs with *Yersinia* and measured the amount of IL-1β released into the supernatant 4 h postinoculation. The *Y. pseudotuberculosis* Δyop6 strain induced a low level of IL-1β secretion (Fig. 4B), as previously described (3). A strain lacking the T3SS cargo protein YopK induced significantly more IL-1β, which is also consistent with previous data (26). Importantly, while the translocation-competent Δyop6 Δ*yopN* strain hyperinduced IL-1β secretion to the same level as the *yopK*-minus strain, the *Y. pseudotuberculosis* Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strain did not induce detectable IL-1β secretion (Fig. 4B). These data indicate that T3SS-mediated pore formation in macrophages is insufficient to trigger this host signaling pathway and that potassium efflux alone is not likely to trigger IL-1β secretion during infection with *Y. pseudotuberculosis*.

Previous work has demonstrated that pore-forming, translocation-competent *Y. pseudotuberculosis* cells induce Egr1 and TNF-α expression in a TLR-independent manner (6). To determine if pore formation alone is sufficient to induce TLR-independent Egr1 expression, we infected $MyD88^{-/-}$ $Trif^{-/-}$ immortalized BMDMs, which are TLR deficient, with *Yersinia* and measured transcription of Egr1 and TNF-α 2 h postinoculation. The *Y. pseudotuberculosis* Δyop6 Δ*yopN* strain induced a 24-fold increase in Egr1 transcription and a 7-fold increase in TNF-α transcription, while infection with the *yopB*-minus strain did not induce significant Egr1 or TNF-α transcription (Fig. 4C and D). Importantly, the *Y. pseudotuberculosis* Δyop6 Δ*yopN yopD*$_{\Delta TM}$ strain also did not induce significant Egr1 expression (Fig. 4C). While the Δyop6 Δ*yopN yopD*$_{\Delta TM}$ mutant triggered 2-fold more TNF-α expression in macrophages compared to the Δyop6 Δ*yopN* Δ*yopB* strain, this was not statistically significant (Fig. 4D,

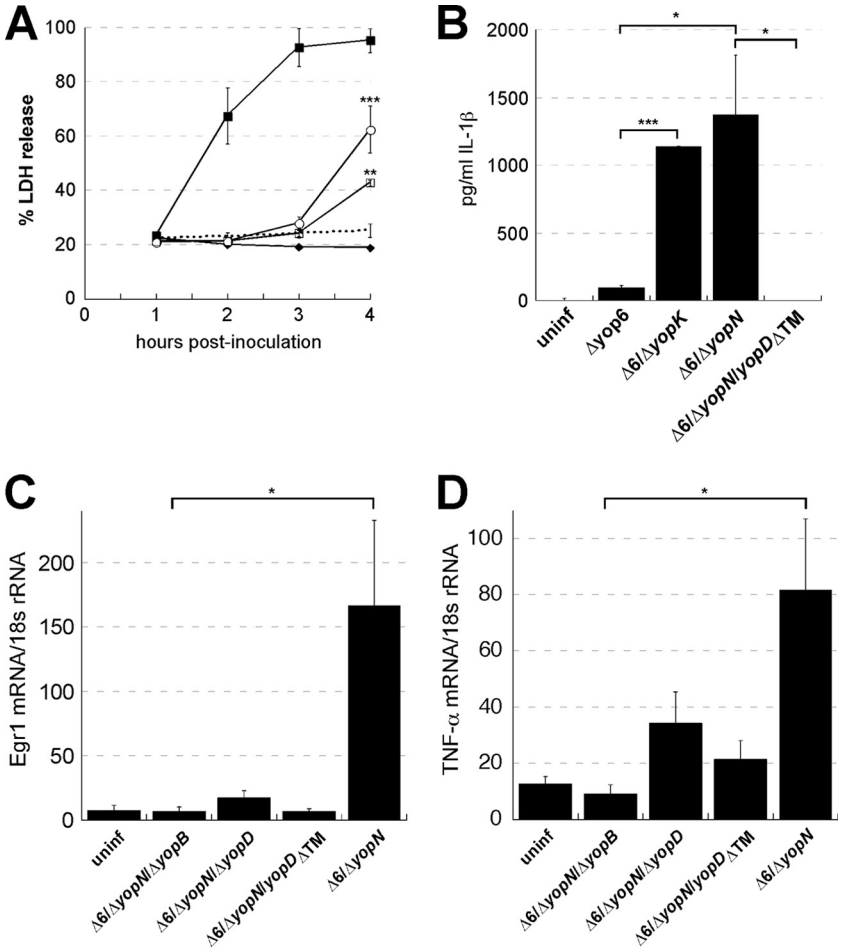

FIG 4 Analysis of LDH release, IL-1β secretion, and Egr1 and TNF-α expression in *Y. pseudotuberculosis*-infected macrophages. (A) Immortalized C57BL/6 BMDMs were infected with *Y. pseudotuberculosis* at an MOI of 15. At various times postinoculation, supernatants were analyzed for lactate dehydrogenase (LDH). The amount of LDH released from freeze-thaw lysis of the cell monolayer was set at 100%. Dotted line, uninfected; diamonds, Δyop6 Δ*yopN* Δ*yopB* strain; circles, Δyop6 Δ*yopN* Δ*yopD* strain; closed squares, Δyop6 Δ*yopN* strain; open squares, Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain. The averages ± SEM of four independent experiments are shown. **, $P \leq 0.005$, and ***, $P \leq 0.0005$, as determined by one-way ANOVA followed by Bonferroni's *post hoc* test, where each indicated group was compared to the appropriate negative and positive controls (Δyop6 Δ*yopN* Δ*yopB* and Δyop6 Δ*yopN* strains). (B) Primary C57BL/6 BMDMs were infected with *Y. pseudotuberculosis* at an MOI of 20 for 4 h, and the amount of IL-1β in the supernatant was measured by ELISA. The averages ± SEM of three independent experiments are shown. *, $P \leq 0.05$, and ***, $P \leq 0.0005$, as determined by one-way ANOVA followed by Bonferroni's *post hoc* test, where each indicated group was compared to the appropriate negative and positive controls (uninfected [uninf] and Δyop6 or Δyop6 Δ*yopN* strain). (C and D) Immortalized $MyD88^{-/-}$ $Trif^{-/-}$ BMDMs were infected with *Y. pseudotuberculosis* at an MOI of 10 for 2 h, and the amounts of TNF-α and Egr1 mRNA were measured by qPCR and compared to that of the 18S rRNA control. The average ± SEM of the ratio of mRNA to 18S rRNA from four independent experiments is shown. *, $P \leq 0.05$, as determined by one-way ANOVA followed by Bonferroni's *post hoc* test (comparing the Δyop6 Δ*yopN* Δ*yopB*, Δyop6 Δ*yopN*, and Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strains).

$P = 1$). Furthermore, the Δyop6 Δ*yopN* $yopD_{\Delta TM}$ mutant did not induce more TNF-α or Egr1 expression than the *yopB*-minus strain even at a high multiplicity of infection (MOI of 100) (V. Auerbuch, unpublished results). These data suggest that T3SS-mediated pore formation alone cannot trigger TLR-independent expression of the stress-associated transcription factor Egr1 or the inflammatory cytokine TNF-α.

DISCUSSION

This study reports on the identification of a *Y. pseudotuberculosis* Δyop6 Δ*yopN* $yopD_{\Delta TM}$ mutant capable of forming T3SS-mediated pores in macrophages but lacking the ability to translocate T3SS cargo inside target host cells. Pores inserted into macrophages by the Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain formed with delayed kinetics and were smaller in size than wild-type T3SS pores (1.3 to 3.8 nm compared to 3.8 to 5 nm). However, while clear pore formation in macrophages was measured by two independent assays, no macrophage response associated with innate immune recognition of the T3SS was detectable other than delayed cell death. These results suggest that T3SS-mediated pore formation in the absence of translocation is insufficient to trigger T3SS recognition. However, because the Δyop6 Δ*yopN* $yopD_{\Delta TM}$ strain formed pores that were measurably different than those inserted by a wild-type YopB-YopD translocon, we cannot rule out that the wild-type YopB-YopD translocon is specifically recognized by macrophages. Based on these data, we can revise the possible models for how the *Yersinia* T3SS might trigger inflammation in macrophages: (i) innate

immune stimuli gain access to the host cytoplasm through the YopBD pore, and/or (ii) the wild-type YopB-YopD translocon stimulates an innate immune response. Further experiments identifying *Yersinia* T3SS cargo distinct from YopHEMOJTNK will refine our understanding of how the T3SS is recognized by mammalian cells.

Y. enterocolitica expressing wild-type YopB and an allele of YopD lacking amino acids 121 to 165, which encompasses the predicted transmembrane domain (TM) ($\Delta yopD_{TM}$), retained the ability to insert ion-conducting channels into planar lipid bilayers (46). In contrast, *yopB*-minus *Y. enterocolitica* did not display channel activity. These data are consistent with our results showing that *Y. pseudotuberculosis* expressing $YopD_{\Delta TM}$ allowed both BCECF release and EtBr entry inside macrophages, while *yopB*-minus *Yersinia* did not (Fig. 1 and 2). Matteï et al. recently proposed a so-called three-tiered ring model to describe the putative structure of the LcrV-YopD-YopB translocon complex (42). As YopD has been shown to bind both LcrV and YopB, Matteï et al. suggested that a YopD homo-oligomeric ring is superficially associated with the host membrane but links the LcrV needle tip with a YopB homo-oligomeric ring stably inserted in the host membrane (42). Olsson et al. (34) showed that the YopD predicted TM domain is not required for inserting pores inside RBCs, but is required to support translocation of T3SS cargo inside nucleated cells. Our study further shows that the YopD predicted TM domain is not required to insert 1.3- to 3.8-nm pores inside macrophages, a pore size that is slightly smaller than wild-type pores, with somewhat delayed kinetics (Fig. 1B and C). These data suggest that the association of the YopD oligomer with the host membrane is essential for translocation and contributes to pore formation kinetics and size. In contrast, full deletion of YopD led to no statistically significant macrophage pore formation or pore formation that was below the limit of detection (Fig. 1C and 2B). This suggests that the YopD-YopB interaction may be important for insertion of a YopB oligomer capable of robust pore formation. Further high-resolution imaging studies are needed to test this and other aspects of the three-tiered ring model.

Pyroptosis is a form of caspase-1-dependent, lytic host cell death associated with secretion of inflammatory cytokines, such as IL-1β (47). The ability of *Y. pseudotuberculosis* to trigger pyroptosis in macrophages is dependent on the T3SS but independent of YopHEMOJT (7). Caspase-1 activation by *Yersinia* is dependent on both Nlrc4 and Nlrp3 inflammasome components, although Nlrp3 seems to play the more dominant role (26). Recently, NLRP12 has also been implicated in stimulating IL-1β secretion after *Y. pestis* infection, but the NLPR12 ligand is unknown (29). Nlrc4 recognition of the *Yersinia* T3SS is independent of flagellin (7). Instead, recent evidence suggests that a structural component of the *Salmonella* T3SS, called the PrgJ inner rod protein, is recognized through an Nlrc4-dependent pathway (23, 24), and the *Y. pseudotuberculosis* PrgJ homologue, YscI, activates the Nlrc4 inflammasome (E. Kofoede and R. Vance, personal communication). It is much less clear how the *Yersinia* T3SS activates Nlrp3, which is activated by a broad range of stimuli, including pore-forming toxins (3, 25). Efflux of potassium from host cells through pores has been postulated to be important in Nlrp3 activation (25, 48–50). Shin and Cornelis previously correlated the ability of *Y. enterocolitica* to form pores in host cells with the ability to trigger IL-1β secretion (8). However, the bacterial strains which were able to form pores and trigger IL-1β release in their study also had the capacity to translocate T3SS cargo inside host cells (8), raising the possibility that T3SS-mediated translocation, not pore formation itself, could trigger Nlrp3 activation. Indeed, our study indicates that a *Y. pseudotuberculosis* strain capable of forming 1.3- to 3.8-nm pores in macrophages, but not of translocating T3SS cargo, did not trigger IL-1β secretion or rapid host cell death. These pores should be capable of releasing potassium ions, although we were unable to measure potassium efflux stemming from even wild-type YopBD-mediated pore formation (data not shown). As reported previously (26, 51), inhibition of potassium efflux by addition of excess extracellular potassium to *Y. pseudotuberculosis*-infected macrophages blocked IL-1β release (W. Adams and V. Auerbuch, unpublished data). Taken together, these data suggest that, while potassium efflux plays a role in T3SS-induced IL-1β secretion, the underlying mechanism of Nlrp3 activation may be translocation of unknown T3SS cargo inside host cells. Alternatively, it is possible that a wild-type YopB-YopD complex is required to activate Nlrp3.

Y. pseudotuberculosis expressing YopB was previously shown to trigger NF-κB and mitogen-activated protein (MAP) kinase activation and IL-8 secretion in HeLa cells, while a YopB mutant with a disruption in one of the two YopB TM domains was defective in inducing this host signaling (27, 28). A *Y. pseudotuberculosis* mutant expressing YopD harboring an early frameshift mutation in the middle of the predicted TM domain retained the ability to trigger IL-8 secretion (27). The authors of these reports suggested that YopB membrane insertion led to activation of the MAP kinase pathway. In the present study, we show that *Y. pseudotuberculosis* lacking the YopD predicted transmembrane domain, but secreting wild-type YopB, does not trigger TLR-independent Egr1 expression in macrophages (Fig. 4C). Egr1 expression has been shown to be induced by the p38 MAP kinase pathway in response to cell stress (52, 53). It is possible that YopB membrane insertion triggers MAP kinase signaling in macrophages as it does in HeLa cells but that this leads to expression of genes distinct from Egr1. However, YopB membrane insertion may not trigger a response in macrophages. Further work analyzing the impact of the T3SS on macrophages versus intestinal epithelial cells, both of which are relevant to the enteropathogenic *Yersinia* infection cycle, will be important for understanding the impact of the innate immune response to the T3SS on gut inflammation and bacterial virulence.

ACKNOWLEDGMENTS

This work was supported by UCSC startup funds (V.A.). W.A. was supported by the Eugene Cota-Robles Fellowship Program and by the UCSC CBSE Research Mentoring Institute.

We are grateful to Joanne Engel, Alison Davis, and Halie Miller for critical reading of the manuscript. We thank Melanie Marketon for the YopM-Bla plasmid, Molly Bergman for the Δ*yopK* suicide plasmid, Walter Bray for help with microscopy, Alison Davis for technical advice on the hemolysis assay, Doug Golenbock for the immortalized BMDM cell lines, and Jessica Lasky-Su for guidance on statistical analysis.

REFERENCES

1. **Ting JP, Duncan JA, Lei Y.** 2010. How the noninflammasome NLRs function in the innate immune system. Science **327**:286–290.
2. **Kawai T, Akira S.** 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity **34**:637–650.

3. **Schroder K, Tschopp J.** 2010. The inflammasomes. Cell **140**:821–832.
4. **Vance RE, Isberg RR, Portnoy DA.** 2009. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. Cell Host Microbe **6**:10–21.
5. **Troisfontaines P, Cornelis GR.** 2005. Type III secretion: more systems than you think. Physiology (Bethesda) **20**:326–339.
6. **Auerbuch V, Golenbock DT, Isberg RR.** 2009. Innate immune recognition of *Yersinia pseudotuberculosis* type III secretion. PLoS Pathog. **5**:e1000686. doi:10.1371/journal.ppat.1000686.
7. **Bergsbaken T, Cookson BT.** 2007. Macrophage activation redirects *yersinia*-infected host cell death from apoptosis to caspase-1-dependent pyroptosis. PLoS Pathog. **3**:e161. doi:10.1371/journal.ppat.0030161.
8. **Shin H, Cornelis GR.** 2007. Type III secretion translocation pores of *Yersinia enterocolitica* trigger maturation and release of pro-inflammatory IL-1beta. Cell. Microbiol. **9**:2893–2902.
9. **Miao EA, Ernst RK, Dors M, Mao DP, Aderem A.** 2008. *Pseudomonas aeruginosa* activates caspase 1 through Ipaf. Proc. Natl. Acad. Sci. U. S. A. **105**:2562–2567.
10. **de Grado M, Rosenberger CM, Gauthier A, Vallance BA, Finlay BB.** 2001. Enteropathogenic *Escherichia coli* infection induces expression of the early growth response factor by activating mitogen-activated protein kinase cascades in epithelial cells. Infect. Immun. **69**:6217–6224.
11. **Lavoie EG, Wangdi T, Kazmierczak BI.** 2011. Innate immune responses to *Pseudomonas aeruginosa* infection. Microbes Infect. **13**:1133–1145.
12. **Cornelis GR.** 2006. The type III secretion injectisome. Nat. Rev. Microbiol. **4**:811–825.
13. **Durand EA, Maldonado-Arocho FJ, Castillo C, Walsh RL, Mecsas J.** 2010. The presence of professional phagocytes dictates the number of host cells targeted for Yop translocation during infection. Cell. Microbiol. **12**:1064–1082.
14. **Marketon MM, Depaolo RW, Debord KL, Jabri B, Schneewind O.** 2005. Plague bacteria target immune cells during infection. Science **309**:1739–1741.
15. **Koberle M, Klein-Gunther A, Schutz M, Fritz M, Berchtold S, Tolosa E, Autenrieth IB, Bohn E.** 2009. *Yersinia enterocolitica* targets cells of the innate and adaptive immune system by injection of Yops in a mouse infection model. PLoS Pathog. **5**:e1000551. doi:10.1371/journal.ppat.1000551.
16. **Viboud GI, Bliska JB.** 2005. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu. Rev. Microbiol. **59**:69–89.
17. **Hamad MA, Nilles ML.** 2007. Roles of YopN, LcrG and LcrV in controlling Yops secretion by *Yersinia pestis*. Adv. Exp. Med. Biol. **603**:225–234.
18. **Williams AW, Straley SC.** 1998. YopD of *Yersinia pestis* plays a role in negative regulation of the low-calcium response in addition to its role in translocation of Yops. J. Bacteriol. **180**:350–358.
19. **Cambronne ED, Schneewind O.** 2002. *Yersinia enterocolitica* type III secretion: *yscM1* and *yscM2* regulate *yop* gene expression by a posttranscriptional mechanism that targets the 5′ untranslated region of *yop* mRNA. J. Bacteriol. **184**:5880–5893.
20. **Anderson DM, Ramamurthi KS, Tam C, Schneewind O.** 2002. YopD and LcrH regulate expression of *Yersinia enterocolitica* YopQ by a posttranscriptional mechanism and bind to *yopQ* RNA. J. Bacteriol. **184**:1287–1295.
21. **Sun YH, Rolan HG, Tsolis RM.** 2007. Injection of flagellin into the host cell cytosol by *Salmonella enterica* serotype Typhimurium. J. Biol. Chem. **282**:33897–33901.
22. **Miao EA, Mao DP, Yudkovsky N, Bonneau R, Lorang CG, Warren SE, Leaf IA, Aderem A.** 2010. Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. Proc. Natl. Acad. Sci. U. S. A. **107**:3076–3080.
23. **Kofoed EM, Vance RE.** 2011. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature **477**:592–595.
24. **Zhao Y, Yang J, Shi J, Gong YN, Lu Q, Xu H, Liu L, Shao F.** 2011. The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature **477**:596–600.
25. **Brodsky IE, Monack D.** 2009. NLR-mediated control of inflammasome assembly in the host response against bacterial pathogens. Semin. Immunol. **21**:199–207.
26. **Brodsky IE, Palm NW, Sadanand S, Ryndak MB, Sutterwala FS, Flavell RA, Bliska JB, Medzhitov R.** 2010. A *Yersinia* effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. Cell Host Microbe **7**:376–387.
27. **Viboud GI, So SS, Ryndak MB, Bliska JB.** 2003. Proinflammatory signalling stimulated by the type III translocation factor YopB is counteracted by multiple effectors in epithelial cells infected with *Yersinia pseudotuberculosis*. Mol. Microbiol. **47**:1305–1315.
28. **Ryndak MB, Chung H, London E, Bliska JB.** 2005. Role of predicted transmembrane domains for type III translocation, pore formation, and signaling by the *Yersinia pseudotuberculosis* YopB protein. Infect. Immun. **73**:2433–2443.
29. **Vladimer GI, Weng D, Paquette SW, Vanaja SK, Rathinam VA, Aune MH, Conlon JE, Burbage JJ, Proulx MK, Liu Q, Reed G, Mecsas JC, Iwakura Y, Bertin J, Goguen JD, Fitzgerald KA, Lien E.** 2012. The NLRP12 inflammasome recognizes *Yersinia pestis*. Immunity **37**:96–107.
30. **Bliska JB, Guan KL, Dixon JE, Falkow S.** 1991. Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. Proc. Natl. Acad. Sci. U. S. A. **88**:1187–1191.
31. **Balada-Llasat JM, Mecsas J.** 2006. *Yersinia* has a tropism for B and T cell zones of lymph nodes that is independent of the type III secretion system. PLoS Pathog. **2**:e86. doi:10.1371/journal.ppat.0020086.
32. **Andrews HL, Vogel JP, Isberg RR.** 1998. Identification of linked *Legionella pneumophila* genes essential for intracellular growth and evasion of the endocytic pathway. Infect. Immun. **66**:950–958.
33. **Merriam JJ, Mathur R, Maxfield-Boumil R, Isberg RR.** 1997. Analysis of the *Legionella pneumophila fliI* gene: intracellular growth of a defined mutant defective for flagellum biosynthesis. Infect. Immun. **65**:2497–2501.
34. **Olsson J, Edqvist PJ, Broms JE, Forsberg A, Wolf-Watz H, Francis MS.** 2004. The YopD translocator of *Yersinia pseudotuberculosis* is a multifunctional protein comprised of discrete domains. J. Bacteriol. **186**:4110–4123.
35. **Francis MS, Wolf-Watz H.** 1998. YopD of *Yersinia pseudotuberculosis* is translocated into the cytosol of HeLa epithelial cells: evidence of a structural domain necessary for translocation. Mol. Microbiol. **29**:799–813.
36. **Dewoody R, Merritt PM, Houppert AS, Marketon MM.** 2011. YopK regulates the *Yersinia pestis* type III secretion system from within host cells. Mol. Microbiol. **79**:1445–1461.
37. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academies Press, Washington, DC.
38. **Davis AJ, Mecsas J.** 2007. Mutations in the *Yersinia pseudotuberculosis* type III secretion system needle protein, YscF, that specifically abrogate effector translocation into host cells. J. Bacteriol. **189**:83–97.
39. **Marenne MN, Journet L, Mota LJ, Cornelis GR.** 2003. Genetic analysis of the formation of the Ysc-Yop translocation pore in macrophages by *Yersinia enterocolitica*: role of LcrV, YscF and YopN. Microb. Pathog. **35**:243–258.
40. **Dacheux D, Goure J, Chabert J, Usson Y, Attree I.** 2001. Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages. Mol. Microbiol. **40**:76–85.
41. **Kirby JE, Vogel JP, Andrews HL, Isberg RR.** 1998. Evidence for pore-forming ability by *Legionella pneumophila*. Mol. Microbiol. **27**:323–336.
42. **Mattei PJ, Faudry E, Job V, Izore T, Attree I, Dessen A.** 2011. Membrane targeting and pore formation by the type III secretion system translocon. FEBS J. **278**:414–426.
43. **Faudry E, Vernier G, Neumann E, Forge V, Attree I.** 2006. Synergistic pore formation by type III toxin translocators of *Pseudomonas aeruginosa*. Biochemistry **45**:8117–8123.
44. **Viboud GI, Bliska JB.** 2002. Measurement of pore formation by contact-dependent type III protein secretion systems. Methods Enzymol. **358**:345–350.
45. **Sorg I, Goehring UM, Aktories K, Schmidt G.** 2001. Recombinant *Yersinia* YopT leads to uncoupling of RhoA-effector interaction. Infect. Immun. **69**:7535–7543.
46. **Tardy F, Homble F, Neyt C, Wattiez R, Cornelis GR, Ruysschaert JM, Cabiaux V.** 1999. *Yersinia enterocolitica* type III secretion-translocation system: channel formation by secreted Yops. EMBO J. **18**:6793–6799.
47. **Bergsbaken T, Fink SL, Cookson BT.** 2009. Pyroptosis: host cell death and inflammation. Nat. Rev. Microbiol. **7**:99–109.
48. **Walev I, Reske K, Palmer M, Valeva A, Bhakdi S.** 1995. Potassium-inhibited processing of IL-1 beta in human monocytes. EMBO J. **14**:1607–1614.
49. **Saleh M, Green DR.** 2007. Caspase-1 inflammasomes: choosing between death and taxis. Cell Death Differ. **14**:1559–1560.
50. **Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J.** 2007.

Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ. **14**:1583–1589.

51. **Zheng Y, Lilo S, Brodsky IE, Zhang Y, Medzhitov R, Marcu KB, Bliska JB.** 2011. A Yersinia effector with enhanced inhibitory activity on the NF-kappaB pathway activates the NLRP3/ASC/caspase-1 inflammasome in macrophages. PLoS Pathog. **7**:e1002026. doi:10.1371/journal.ppat.1002026.
52. **Rolli M, Kotlyarov A, Sakamoto KM, Gaestel M, Neininger A.** 1999. Stress-induced stimulation of early growth response gene-1 by p38/stress-activated protein kinase 2 is mediated by a cAMP-responsive promoter element in a MAPKAP kinase 2-independent manner. J. Biol. Chem. **274**:19559–19564.
53. **Lim CP, Jain N, Cao X.** 1998. Stress-induced immediate-early gene, egr-1, involves activation of p38/JNK1. Oncogene **16**:2915–2926.