Injection of T3SS effectors not resulting in invasion is the main targeting mechanism of *Shigella* toward human lymphocytes

Laurie Pinaud\(^{a,b}\), Fatoumata Samassa\(^{a,b}\), Ziv Porat\(^{c}\), Mariana L. Ferrari\(^{a,b}\), Ilia Belotserkovsky\(^{a,b}\), Claude Parsot\(^{a,b}\), Philippe J. Sansonetti\(^{a,b,d,1}\), François-Xavier Campbell-Valois\(^{a,b,2}\), and Armelle Phalipon\(^{a,b,1}\)

*Molecular Microbial Pathogenesis Unit, Department of Cellular Biology of Infection, Institut Pasteur, 75724 Paris, France; \(^{b}\)INSERM U1202, 75015 Paris, France; \(^{c}\)Flow Cytometry Unit, Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot 7610001, Israel; and \(^{d}\)Microbiologie et Maladies Infectieuses, Collège de France, 75005 Paris, France

Contributed by Philippe J. Sansonetti, July 25, 2017 (sent for review May 10, 2017; reviewed by Gad Frankel, Ilan Rosenshine, and Michael N. Starnbach)

The enteroinvasive bacterium *Shigella* is a facultative intracellular bacterium known, in vitro, to invade a large diversity of cells through the delivery of virulence effectors into the cell cytoplasm via a type III secretion system (T3SS). Here, we provide evidence that the injection of T3SS effectors does not necessarily result in cell invasion. Indeed, we demonstrate through optimization of a T3SS injection reporter that effector injection without subsequent cell invasion, termed the injection-only mechanism, is the main strategy used by *Shigella* to target human immune cells. We show that in vitro-activated human peripheral blood B, CD4\(^+\) T, and CD8\(^+\) T lymphocytes as well as switched memory B cells are mostly targeted by the injection-only mechanism. B and T lymphocytes residing in the human colonic lamina propria, encountered by *Shigella* upon its crossing of the mucosal barrier, are also mainly targeted by injection-only. These findings reveal that cells refractory to invasion can still be injected, thus extending the panel of host cells manipulated to the benefit of the pathogen. Future analysis of the functional consequences of the injection-only mechanism toward immune cells will contribute to the understanding of the priming of adaptive immunity, which is known to be altered during the course of natural *Shigella* infection.

Shigella \| T3SS \| bacterial effectors \| human lymphocytes \| host-pathogen cross talk

The Gram-negative enteroinvasive bacteria *Shigella* spp. are responsible for bacillary dysentery, an acute rectocolitis causing high levels of morbidity and mortality predominantly in children under 5 y old in low- and middle-income countries (1). *Shigella* pathogenicity relies on a type III secretion system (T3SS), a device delivering bacterial effectors into the host cell cytoplasm that interfere with intracellular signaling pathways. In intestinal epithelial cells, these effectors manipulate the host cell cytoskeleton to promote bacterial internalization and reprogram cell gene expression to modulate the host innate inflammatory response, which is key for efficient establishment of *Shigella* infection (2). To understand why several rounds of *Shigella* infection induce only a short-term protective humoral immunity and thus overcome the challenges of vaccine development (3, 4), a better knowledge of *Shigella*-immune cell interactions is necessary. Over the last years, *Shigella* has been shown to not only target innate immune cells (5) but also to directly impact adaptive immune cells through mechanisms dependent upon its T3SS (6–8). Our previous studies of *Shigella*-lymphocytes cross talks suggested that, besides invasion of these cells, injection of *Shigella* T3SS effectors not resulting in invasion might also occur (6). Direct visualization of T3SS effector injection can be assessed by taking advantage of a fluorescence resonance energy transfer (FRET)-based \(\beta\)-lactamase assay. This translocation assay originally reported to monitor enteropathogenic Escherichia coli effector translocation (9, 10) is based on reporter bacteria expressing an effector translationally fused to the TEM-1 \(\beta\)-lactamase. When injected into cells loaded with the FRET substrate CCF2-AM (11), the \(\beta\)-lactamase cleaves the cephalosporin core of CCF2-AM, inducing a detectable shift in fluorescence from green to blue (Fig. S1). By using this assay, we showed injection of the *Shigella* T3SS effector IpglD into human T lymphocytes while using a high multiplicity of infection (MOI) and preventing invasion with the actin polymerization inhibitor cytochalasin D (6). These findings suggested that injection of T3SS effectors might indeed be uncoupled from cellular invasion. The purpose of the current study was thus to assess the different targeting mechanisms used by *Shigella* while interacting with human lymphocytes, including the formal demonstration of injection occurring in the absence of invasion. To document these events in a more physiological setting (use of a lower MOI and no inhibition of bacterial invasion), we improved both parts of the injection reporter, identifying a better-secreted T3SS effector and a more efficient \(\beta\)-lactamase enzyme. In addition, direct distinction between invaded vs. “injected-only” cells was implemented by using imaging flow cytometry analysis combined with *Shigella* expressing the DsRed fluorescent protein. This strategy successfully demonstrated that the predominant *Shigella*-mediated mechanism to target primary human lymphocytes is indeed “injection-only.”

Significance

Invasion of human intestinal epithelial cells resulting from the delivery of bacterial effectors into the host cell cytoplasm via a type III secretion system (T3SS) is the hallmark of *Shigella*, the causal agent of bacillary dysentery. Here, we provide evidence that human lymphocytes are mainly targeted by injection of T3SS effectors not resulting in cell invasion. These findings highlight the diversity of mechanisms triggered by *Shigella* to enlarge the panel of targeted cells and thus counteract host immunity, including adaptive immune responses. The potential impact of these data on vaccine design, in particular for live-attenuated vaccine candidates, deserves further investigation.

Author contributions: L.P., C.P., P.J.S., F.-X.C.-V., and A.P. designed research; M.L.F. and I.B. gave advice and ideas to complete the study; L.P. performed research; L.P., F.S. and Z.P. analyzed data; and L.P. and A.P. wrote the paper.

Reviewers: G.F., Imperial College London; I.R., The Hebrew University; and M.N.S., Harvard Medical School.

The authors declare no conflict of interest.

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1To whom correspondence may be addressed. Email: philippe.sansonetti@pasteur.fr or armelle.phalipon@pasteur.fr.

2Present address: Department of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, ON, Canada K1N 6N5.

This article contains supporting information online at www.pnas.orglookup?uppl doi:10.1073/pnas.1707098114/DCSupplemental.

9554–9559 | PNAS | September 12, 2017 | vol. 114 | no. 37

www.pnas.org/cgi/doi/10.1073/pnas.1707098114
Results

Optimization of the Injection Reporter. Both parts of the injection reporter were optimized by identifying a more efficiently secreted T3SS effector and a more active β-lactamase enzyme. First, a panel of T3SS effectors were expressed as chimeric proteins fused to the β-lactamase TEM-1 (bla) in the constitutively secreting ipaD Shigella mutant strain (12), and secretion of most of the full-length chimeric proteins was confirmed in culture supernatants (Fig. 1A). The β-lactamase activity measured in culture supernatants, using the colorimetric substrate nitrocefin, was shown to be optimal with the enzyme fused to the first 80 aa of the T3SS effector OspD1, named OspD1sh (Fig. 1B). Interestingly, the enzymatic activity was 35 times higher in the supernatants of ipaD strains expressing OspD1sh-bla compared with full-length OspD1-bla (Fig. 1B). Exchange of the methionine-31 residue to leucine (M31L) abolished an alternative translation start site in the OspD1sh-bla construct, resulting in only the full-length fusion protein being expressed (Fig. 1A), with no impact on the enzymatic activity detected in ipaD supernatants compared with strains expressing OspD1sh-bla (Fig. 1B). As a negative control, the sequence coding for residues 2–30 of the OspD1sh-M31L-bla construct was deleted, abolishing secretion of the resulting OspD1ctrl-bla chimeric protein into ipaD supernatants (13) (Fig. 1A and B). Noteworthy, the enzymatic activity measured in the supernant of ipaD bacteria expressing OspD1sh-M31L-bla was 40 times higher compared with the previously used IpgD-bla reporter construct (6) (Fig. S2A). Moreover, detection of T3SS-mediated effector translocation into CCF2-AM–loaded Jurkat T lymphocytes was significantly improved upon infection with WT Shigella expressing OspD1sh-M31L-bla compared with IpgD-bla (Fig. S2 B–E).

Since the core of CCF2 is composed of a cephalosporin, a class of antibiotics most efficiently processed by class C β-lactamases (14), members of this group were selected and their coding sequence fused in 3′ of the sequence coding for OspD1sh-M31L. The enzymatic activity measured upon incubation of CCF2 with supernatants of ipaD strains expressing these class C β-lactamases–based chimeric proteins was only 4–15% of that measured in supernatants of strains expressing TEM-1–based chimeric proteins (Fig. S3). OspD1sh-M31L was then transfectedentially fused to the TEM-1 natural variant TEM-3 (also known as CTX-1) displaying increased resistance to cephalosporins (15). In addition, the point mutation M182T known to stabilize TEM β-lactamases was introduced (16). The resulting construct displayed a 60% increase in activity toward CCF2 in ipaD supernatants compared with strains expressing the TEM-1–based chimeric protein, and was thus selected (Fig. 1C). Last, to discriminate between T3SS effector translocation coupled to invasion as opposed to injection-only, the sequence coding for theDsRed fluorescent protein was inserted into the reporter plasmid under the control of a constitutive promoter (Fig. S4A). No impact on the detection of reporter translocation into Jurkat T lymphocytes was observed (Fig. 1D). Hereafter, plasmids encoding DsRed and either the T3SS injection reporter OspD1sh-M31L-TEM3-M182T or its control counterpart OspD1ctrl-TEM3-M182T are referred to as Rep-bla and Ctrl-bla, respectively. WT strains expressing Rep-bla or Ctrl-bla could be used to induce replicate and spread within epithelial cell monolayers to a similar extent compared with the WT strain expressing DsRed only (Fig. S4B).

T3SS Injection Occurs Independently from Invasion in Jurkat T Lymphocytes. Occurrence of invasion vs. injection-only events was assessed by using imaging cytometry, a technology combining flow-cytometry with single-cell epifluorescence microscopy. By applying the analysis strategy described in Fig. S5 to infection of the Jurkat human T-lymphocyte cell line, three types of targeting mechanisms were identified within WT-Rep-bla–infected cells (Fig. 2A and B) and their respective proportion quantified (Fig. 2B). While confirming invasion (6), injection-only appeared to be a major targeting strategy toward these cells. Among targeted cells, those with a bacterium associated to their plasma membrane (PM-association) were considered a separated category of events since the outcome of bacterial attachment to cell surface in terms of invasion vs. injection-only is unpredictable. Similar results were obtained using multiplicity of infections (MOIs) of 50 and 10, demonstrating that the number of targeted cells had no impact on targeting mechanism proportions. As previously checked for epithelial cells, the invasiveness of WT-Rep-bla was compared with that of the control strains. Its invasion rate was about twofold lower than that of WT-Ctrl-bla or WT-DsRed (Fig. S6A). Similar results were obtained with different sources of human primary cells, that is, blood lymphocytes (Fig. S6B) and lamina propria cells from colonic explants (Fig. S6C). Consequently, quantification of injection-only vs. invasion mechanisms was considered to be relevant only when the proportion of targeted cells exceeded at least twice that of invaded cells.

In Vitro-Activated Primary Blood Lymphocytes and Switched Memory B Cells Are Largely Targeted by Injection-Only. To investigate Shigella targeting of primary lymphocytes, peripheral blood
mononuclear cells (PBMCs) and, subsequently, B, CD4^+ T, and CD8^+ T lymphocytes were isolated from different donors. Lymphocyte viability and purity were systematically assessed (Fig. S7 A–C). For each donor, some cells were kept nonactivated while others were activated in vitro using interleukin-2 (IL-2) and protein A from *Staphylococcus aureus* for B lymphocytes (SAC) or phorbol 12-myristate 13-acetate (PMA) for T lymphocytes. Activation efficiency and cell viability were verified before infection (Fig. S7 B and C).

By implementing an analysis similar to that applied to the Jurkat cell line (Fig. S5), we showed that 30–60% of in vitro-activated primary B, CD4^+ T, and CD8^+ T lymphocytes were targeted upon infection with WT-Rep-bla with a MOI of 10 (Fig. 3A). Regarding nonactivated lymphocytes that were infected the day following isolation, only B cells were significantly targeted by *Shigella* (Fig. 3B), but to a lesser extent than their activated counterparts. Among these, switched memory B cells were preferential targets as opposed to naive B cells (Fig. 3C and Fig. S7 D and E). Overall, incubation with *Shigella* had no impact on blood primary lymphocyte viability during the time frame of the experiment (Fig. S8A). Regarding the absence of detection of nonactivated blood T lymphocytes targeted by *Shigella*, we cannot rule out that minor subsets, such as regulatory CD4^+ T cells, might have been missed due to the analysis performed on all CD4^+ T lymphocytes. Among the blood-derived activated B and T lymphocytes and the switched memory B cells, we further demonstrated that only a minor proportion of targeted cells were invaded, while injection-only constituted the main targeting mechanism (Fig. 3D).

Human Colonic Lamina Propria B and T Lymphocytes Are Almost Exclusively Targeted by Injection-Only. Immune cells isolated from the lamina propria of human colonic explants (Fig. 4A), which are the first cells encountered by *Shigella* upon crossing the colonic epithelium barrier, were investigated. Briefly, the mucosa was dissected away from the muscle layer and epithelial cells were removed through successive EDTA incubations. Lamina propria cells were released upon collagenase treatments before CCF2-AM loading. Cells isolated through this procedure displayed variable viability from donor to donor (usually around 75%; Fig. S9A). Only a minor contamination of epithelial cells (below 2%) was observed while more than 90% of isolated cells were CD45^+ (Fig. S9B), among which the majority was either CD19^+ or CD3^+ (Fig. S9C). Following infection with WT-Rep-bla and WT-Ctrl-bla for 1 h at a MOI of 50, 60% of cells were still alive (Fig. S8B) and 8–14% of live loaded cells were detected as targeted by *Shigella* WT-Rep-bla (Fig. 4B).

The imaging flow cytometry procedure to quantify invaded vs. injected-only events was then implemented with some additional steps to exclude apoptotic, autofluorescent, and misshapen cells after gating on live loaded cells (Fig. S9D). About 50–96% of the *Shigella*-targeted lamina propria lymphocytes were shown to be injected-only (Fig. 4C), including CD19^+ and CD3^+ lymphocytes (Fig. 4D). Altogether, these findings revealed a thus-far-undetected mechanism used by *Shigella* to target host cells.

**Discussion**

In this study, we developed a *Shigella* T3SS-injection reporter tool adapted to the investigation of targeting mechanisms toward immune cells, using imaging flow cytometry to directly visualize invasion vs. injection-only events at the single-cell level. In the field of host–pathogen interactions, imaging flow cytometry has been used so far to study the intracellular fate of bacteria or parasites (17–20). This report describes an experimental strategy to simultaneously distinguish and quantify different *Shigella* targeting mechanisms among a given population of cells. Using this approach, we demonstrated that injection of *Shigella* T3SS effectors into some B- and T-lymphocytes subsets, including those relevant during natural infection of the human colon, does not necessarily result in cell invasion. We propose that this injection-only mechanism is at the core of interactions with cells refractory to invasion, thus extending the panel of host cells manipulated to the benefit of the pathogen. We hypothesize that susceptibility to *Shigella* T3SS effector injection, followed or not by invasion, depends on host cell morphology. This concept is based on our observations using the optimized translocation reporter of a preferential targeting of activated lymphocytes over their nonactivated counterparts, as well as switched memory B cells over naive B cells. Indeed, the amount of cytoskeleton components and the size of the cytoplasmic compartment, which are required for bacterial invasion and replication, are largely reduced in lymphocytes compared with epithelial cells but increase upon their activation (21, 22). In particular, considering the critical role of host cytoskeleton rearrangements for *Shigella*-induced invasion of epithelial cells (23), we speculate that the amount of cytoskeleton components that can be hijacked by injected bacterial effectors is critical for the outcome of T3SS injection. Above the threshold required to trigger the full process of bacterial internalization, T3SS injection results in cellular invasion, while below this threshold injection-only occurs, thus permitting an increased diversity in the nature of targeted cells, beyond those sensitive to invasion.
Thus far, targeting of human colonic lymphocytes by enteropathogenic bacteria has been poorly investigated. Indeed, modulation of B- and T-lymphocyte functions upon infection with Yersinia, Salmonella, EPEC, and EHEC has been studied using in vitro human colon cell lines or in vivo mouse models of infection (24–28). In contrast, our findings constitute a description of primary human colonic lymphocyte interactions with enteropathogenic bacteria.

We consider that the current findings along with our previous report on B lymphocytes (8) leaves the door open to reexamine the current understanding of T3SS-mediated Shigella pathogenicity. We previously reported a Shigella targeting mechanism toward some B-lymphocyte subsets that is independent from both invasion and T3SS effector injection, demonstrating that these cells undergo apoptosis after activation of a TLR2-dependent signaling pathway triggered by the T3SS apparatus needle tip protein IpAD (8). Therefore, we propose a paradigm for Shigella pathogenicity termed the “kiss-and-run” strategy. This depicts the ability of Shigella to manipulate targeted cells via a T3SS-dependent contact, which results in either no T3SS effector delivery into the host cell cytoplasm or their delivery not followed by cell invasion. Long studied for its life cycle as an intracellular pathogen based on its ability to invade almost any cell type in vitro (29, 30), these findings highlight an overlooked aspect of Shigella’s extracellular lifestyle. Considering the large panel of T3SS effectors reported to alter host cell processes (2, 31–33), the impact of injection-only on lymphocyte functions is likely to contribute to the observed impaired priming of host-specific immunity. It is likely that the lack of CD8+ T-cell priming reported using a mouse model of Shigella infection (34) might result from T3SS injection-only occurring in these cells, as reported in this work. The injection-only mechanism may also explain the Shigella-induced modulation of antimicrobial peptide production observed in noninvaded differentiated intestinal epithelial cells (35). Future studies at the single-cell level will probe the impact of the injection-only outcome on immune cells targeted by Shigella within the human colonic mucosa and might significantly impact efforts to develop a Shigella vaccine. Indeed, in particular, the design of live, rationally attenuated, orally administered vaccine candidates will have to take into consideration the so-far-underestimated T3SS activity toward B and T lymphocytes that are key for the priming of efficient protective immunity.

Materials and Methods

Bacterial Strains and Cloning Reagents. The Shigella flexneri 5a (referred to as Shigella or S. flexneri) M90T WT strain (36) and its derivative mutant strain ipaD (37) were grown in tryptic soy broth (TSB), supplemented when appropriate with 10 μg/mL chloramphenicol. Chemically competent Escherichia coli DH5α bacteria (Invitrogen) were used for all cloning steps and grown at 37 °C in yeast tryptone medium (2xYT), supplemented when appropriate with 30 μg/mL chloramphenicol. NucleoSpin Plasmid miniprep, PCR clean-up, and gel extraction kits (Macherey-Nagel), and PureLink HiPure midiprep kit (Invitrogen) were used for DNA preparation. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, Taq DNA polymerase, and Phusion polymerase were purchased from Thermofisher Scientific.

Plasmids. The pBAD-IgpD-TEM1 plasmid was previously described (6). Details of plasmids construction for the obtention of WT-Rep-bla are described in Supporting Information.

Bacterial Lysates and Immunoblotting. Bacterial overnight cultures were subcultured 1/50 in TSB at 37 °C without antibiotics. Subcultures were spun at 9,000 × g for 1 min, and an equivalent amount of bacteria or supernatants was resuspended in Laemmli buffer (Bio-Rad). Boiled lysates were analyzed by SDS/PAGE, and proteins of interest were detected by mouse anti-β-lactamase antibody (Abcam, ab12251; or Novus Biologicals, NB120-12251; 1/1,000) and anti–mouse-HRP antibody (GE Healthcare; NKA931; 1/5,000), using film detection or Amersham Imager 600 (GE Healthcare).

Secretion Assay. Overnight cultures of ipaD bacteria were grown at 30 °C and subcultured 1/50 in TSB at 37 °C for 4 h without antibiotics. Subcultures were spun at 9,000 × g for 1 min, and 100 μL of supernatants at different dilutions were incubated with β-lactamase substrates: 20 μL of nitrocefin (500 μg/mL stock; EMD Millipore) or 20 μL of CF2-FA (25 μM stock; Thermo Fisher Scientific). Hydrolysis of the colorimetric substrate nitrocefin [absorbance, 486 nm (abs486)] and cleavage of the fluorescent substrate CF2-FA [excitation, 405 nm; emission, 460 nm (em460)] were measured over time using TECAN Infinite M200 Pro (LifeSciences). β-Lactamase activity was calculated in equivalent Miller units (mU) as follows: Activity [mU] = 1,000 × (abs486 or em460[sample] – abs486 or em460[TSB])/([TSB]/(time [min] × volume [mL] × OD600[subculture] × sample dilution).

Human Cell Lines. Jurkat T cells (clone E6-1; ATCC TIB-152) were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS (HI-FCS) at 37 °C in 5% CO2 incubator. T7 cells (a clone of Caco-2) were cultured in DMEM, 1 g/L glucose, supplemented with nonessential amino acids, glutamine (all from Gibco), and 20% HI-FCS at 37 °C in 10% CO2 incubator.

Isolation of Primary B and T Lymphocytes from Human Blood. Samples were obtained following patients’ informed consent from Etablissement Français du Sang (study approved by the Institut Pasteur’s ethical and medical committee, agreement HS2015-24009). PBMCs were purified by density separation on Ficoll Paque Plus (GE Healthcare) upon centrifugation at room temperature (RT) for 30 min at 800 × g without brake. Remaining red blood cells (RBCs) were lysed using RBC lysis buffer (Sigma). B, CD4+ T, CD8+ T,
Human colonic lamina propria lymphocytes are preferentially targeted via injection-only upon in vitro infection with Shigella. (A) Confocal single slices of human colon specimen. B (CD20cy, red) and T lymphocytes (CD3, green); DAPI-stained nuclei (gray); phalloidin-associated actin (blue). Arrows denote B- and T-lymphocyte aggregates. (Scale bar: 50 μm.) (B–D) Analysis of CCF2-AM–loaded cells isolated from human colonic lamina propria and infected for 1 h at an MOI of 50 with Shigella WT-Rep-bla or WT-Ctrl-bla. (B) Flow cytometry analysis of targeted cells. Each dot represents one donor. Filled circles: WT-Rep-bla–infected cells; open circles: WT-Ctrl-bla–infected cells; filled squares: uninfected cells. ***P ≤ 0.001 (Student’s t test compared with WT-Ctrl-bla–infected cells). Data are from four independent donors. (C) Imaging cytometry quantification of invaded, injected-only, and PM-associated event proportions among targeted cells. Data from three independent donors are represented (analysis of n=250 targeted cells per donor). (D) Representative images of imaging cytometry acquisition displaying lamina propria injected-only targeted cells with anti-human CD3 or CD19 surface staining.

Isolation of Primary Cells from Human Colonic Specimens. Tissue specimens were obtained following patients’ informed consent from Hôpital Mondor, Gastroenterology Department, Creteil, France (collaboration with A. Amiot and I. Sobhani; study approved by the Institut Pasteur’s ethical and medical committee, agreement 2012-37). Samples were taken at a distance from the tumor in patients undergoing surgery for colon cancer. Details of cell isolation are described in Supporting Information. Antibodies used for purity and targeted cell phenotyping are listed in Table S1.

Immunofluorescence Staining of Colon Specimens. Immunohistochemistry procedures are presented in Supporting Information.

Shigella Infection and Translocation Assay. Bacterial overnight cultures grown at 30 °C were subcultured 1/50 in TSB at 37 °C without antibiotics until early exponential phase was reached. Cells were seeded 1 h before infection in 96-well plates (round bottom) at 3–5 × 10^5 cells per well in translocation assay medium [RPMI 1640 supplemented with 2.5 mM probenecid (VWR) and CCF2-AM (Thermo Fisher Scientific); final concentration of 1 μM was used for Jurkat cells, 2 μM for primary cells]. When indicated, cytochalasin D was used at a final concentration of 1 μM (Sigma-Aldrich). Bacterial subcultures were adjusted in RPMI 1640–probenecid to the indicated MOI, and centrifuged onto the cells at 300 × g and 37 °C for 5 min. Infection was allowed to proceed for indicated times at 37 °C in a 5% CO2 incubator. When indicated, gentamicin was used at 50 μg/mL to kill extracellular bacteria. Following infection, probenecid was kept in all buffers, including during washing steps. To assess viability, cells were washed in cold PBS–probenecid and stained for 30 min on ice with Live/Dead fixable near-infrared dead cell stain kit (Thermo Fisher Scientific). Infected cells were kept on ice in cold PBS–probenecid supplemented with 0.1% BSA for analysis. For imaging cytometry, cells were fixed in 1% PFA–probenecid for 10 min at RT before acquisition.

Flow Cytometry and Imaging Flow Cytometry Data Analysis. Flow cytometry data acquisition was achieved using a FACSCTLL flow cytometer (BD Biosciences) equipped with 405-, 488-, and 633-nm lasers. FlowJo, version 10.0.8, software was used to analyze the data. Dead cells were excluded based on Live/Dead staining (Thermo Fisher Scientific) and/or forward- and side-scatter profiles. A minimum of 10,000 live cells was analyzed per sample. Imaging flow cytometry data acquisition was achieved using an ImageStream X Mk I (Amnis, part of EMD Millipore-Merck) equipped with dual camera and 405-, 488-, 561-, and 633-nm excitation lasers. Samples were acquired at 40x (N.A., 0.75) magnification with the 488-nm laser switched off. A minimum of 5,000 events corresponding to focused, single cells was collected for each sample. Channels used were channels 1 and 9 [brightfield (BF)], channel 3/4 (Shigella–DsRed fluorescence), channels 7 and 8 (cleaved and uncleaved CCF2-AM fluorescence, respectively), channel 11 (staining with Qdot705 fluorophore-conjugated antibodies), and channel 12 (Live/Dead staining fluorescence). Data analysis was performed using the IDEAS, version 6.2, software (Amnis). Masks (areas of interest) and features (calculations made from masks) were generated to give quantitative measurement of the collected images.

Plaque Assay. Plaque assays were performed as described previously (38), using TC7 cells cultured to confluence for 2 wk. Following 2-h infection with 10^8 bacteria at 37 °C in 10% CO2 incubator, an overlay of 0.5% agarose containing 50 μg/mL gentamicin in culture medium was poured into each well after extensive washing of the cell monolayers. Three days later, cells were fixed for 5 min in 100% ethanol and stained for 10 min in Giemsa R solution (RAL Diagnostics; diluted 1/20 in water), and plaques were enumerated.

Data Presentation and Statistical Analysis. Prism 6.0 (GraphPad Software) was used for graphs and statistical analyses. Means and SDs are represented. Unpaired two-tailed Student’s t test was used to compare two groups. The illustrated CSS software (Adobe) was used to assemble figures.

ACKNOWLEDGMENTS. We thank Aurélien Amiot for providing human colonic tissue specimen and Céline Mulet for its characterization by microscopy; Marc Galimand and Delphine Girlich for supplying bacteria strains expressing class C β-lactamases; Giulia Nigro, Daniel Scott-Algara, Asmaa Tazi, and Nicolas Doucet for discussion and advice; Mark Anderson for reviewing the manuscript; the Centre for Human Immunology for the use of their technical facilities; and Florence Videau for her expertise in imaging cytometry. This study was supported by the French Ministry of Higher


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