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Virulent *Shigella flexneri* subverts the host innate immune response through manipulation of antimicrobial peptide gene expression

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Antimicrobial factors are efficient defense components of the innate immunity, playing a crucial role in the intestinal homeostasis and protection against pathogens. In this study, we report that upon infection of polarized human intestinal cells in vitro, virulent *Shigella flexneri* suppress transcription of several genes encoding antimicrobial cationic peptides, particularly the human β-defensin hBD-3, which we show to be especially active against *S. flexneri*. This is an example of targeted survival strategy. We also identify the MxiE bacterial regulator, which controls a regulon encompassing a set of virulence plasmid-encoded effectors injected into host cells and regulating innate signaling, as being responsible for this dedicated regulatory process. In vivo, in a model of human intestinal xenotransplant, we confirm at the transcriptional and translational level, the presence of a dedicated MxiE-dependent system allowing *S. flexneri* to suppress expression of antimicrobial cationic peptides and promoting its deeper progression toward intestinal crypts. We demonstrate that this system is also able to down-regulate additional innate immunity genes, such as the chemokine CCL20 gene, leading to compromised recruitment of dendritic cells to the lamina propria of infected tissues. Thus, *S. flexneri* has developed a dedicated strategy to weaken the innate immunity to manage its survival and colonization ability in the intestine.
Permeabilization of target membranes is the crucial step in defense-mediated antimicrobial activity and cytotoxicity (9). In bacteria, this phenomenon coincides with inhibition of RNA, DNA, protein synthesis, and a decrease in bacterial viability (10).

Antimicrobial factors have been linked to the bridging of innate and acquired immune responses. Indeed, cathelicidin and various β-defensins show chemotactic activity for immune cells such as monocytes, T cells, or immature DCs, and can induce cytokine production by monocytes and epithelial cells (11, 12). Several pieces of evidence indicate that some activities of defensins are receptor mediated, resulting in activation of downstream signaling events. Indeed, it has been demonstrated that the chemotactic activity of hBD-1, -2, and -3 for memory T cells and immature DCs is mediated through binding to the chemokine receptor 6 (CCR6), which is the receptor for the chemokine (C-C motif) ligand 20 (CCL20) (12). Conversely, some molecules with chemotactic properties for immune cells, such as chemokine CCL20, exhibit antimicrobial activity against various Gram-positive and -negative bacteria (13). The rapid expression of antimicrobial factors is thus correlated to the recruitment of adaptive immune cells capable of directing a long-lasting cellular response to pathogens (11).

Among enteric pathogens, virulent S. flexneri are highly contagious Gram-negative enteroinvasive bacteria that cause bacillary dysentery. In malnourished toddlers in the developing world, untreated infections can be fatal. S. flexneri is unable to invade intestinal epithelial cells through the apical surface, but needs to translocate the intestinal epithelium for the development of infection (14). The pathogen translocates through M cells of the follicle-associated epithelium that covers the lymphoid nodules associated with the colonic mucosa. In the subepithelial location, S. flexneri causes apoptosis of resident macrophages, allowing bacterial escape into the tissues and efficient basolateral entry into epithelial cells, followed by cell-cell spread and efficient intracellular growth (15, 16). Subsequent inflammation destroys cohesion of the epithelial barrier, facilitating further invasion of luminal bacteria and propagation of the infection (17). These complex processes are orchestrated by bacterial effectors that are encoded by plasmid-located gene clusters and are injected into cells through a type III secretion system (TTSS) (18). Under conditions of secretion, expression of 12 of these genes is controlled by MxiE, a transcriptional activator of the AraC family (19). MxiE-dependent proteins are thought to be essential for the infection process, as exemplified by OspF and OspG, which negatively regulate the host innate immune response induced by S. flexneri upon invasion of the epithelium (20, 21).

In the course of the invasion of the human intestinal epithelial lining, S. flexneri is confronted by the surface antimicrobial innate defense systems. Recent studies reported that S. flexneri may interfere with this host barrier defense system by down-regulating expression of genes from the innate immune response such as HBD1 and LL37 (22, 23). This immune escape strategy may also be used by other human pathogens, such as Salmonella enterica serovar Typhimurium and Neisseria gonorrhoeae (24, 25). Thus, modulation of the innate defense system may be a general mechanism facilitating invasion of pathogenic bacteria at mucosal surfaces.

In this study, by combining in vitro and in vivo approaches, we clearly demonstrate that S. flexneri actively dampens host innate immune responses by suppressing transcription of specific antimicrobial peptide genes. We also identify the bacterial effectors controlled by the MxiE transcriptional activator as the molecules responsible for this mechanism. In vivo, the strategy used by S. flexneri to modulate transcription of these target genes was followed by a decrease in the production of these epithelial cell-encoded peptides, coupled to a deeper progression of denser population of luminal bacteria toward crypts. We demonstrate that MxiE-dependent effectors are also able to down-regulate additional innate immunity genes, such as chemokine genes, leading to a negative regulation of the DCs recruitment to the lamina propria of infected human tissues. The production of antimicrobial molecules and recruitment of immune cells is one of the major immune responses dedicated to the protection of the mucosal surfaces, thus, this work highlights the strategy developed by S. flexneri to subvert the dynamic defense processes of the intestinal epithelium.

RESULTS
Virulent S. flexneri modulates expression of specific innate immunity genes in vitro
To investigate whether S. flexneri modulates expression of genes involved in the intestinal innate immune response, such as the genes encoding antimicrobial factors, we established an in vitro model of infection based on polarized and differentiated TC7 or HT29 colonic epithelial cells. This model mimics the epithelial lining and allows detection of transcripts for antimicrobial factors whose expression are correlated with the degree of cellular differentiation (26). Infections were performed on cell monolayers for 1 h 30 min with the invasive wild-type S. flexneri strain M90T and its isogenic derivatives: the invasive mxiE mutant, the noninvasive mxiD mutant, and the noninvasive plasmid-cured BS176 strain. The mxiE mutant is impaired for the MxiE transcriptional activator regulating expression of several virulence factors, such as the Osp and IpaH proteins, encoded by the virulence plasmid and injected into cells upon infection, whereas the mxiD mutant is impaired for the assembly of a functional TTSS. The infection was stopped by addition of gentamicin. Host cell RNAs were harvested at 1 h 30 min, 3 h, and 4 h 30 min after infection and analyzed by qRT-PCR for the expression of a selected set of innate immunity genes.

In noninfected cell monolayers, we observed a basal expression of the β-defensins HBD1, HBD2, and HBD3, cathelicidin LL37, chemokine CCL20, and chemokine receptor CCR6-encoding genes. In contrast, we were unable to detect transcription of the β-defensin HBD4 gene in all tested conditions, including infection experiments (unpublished data). Infection of TC7 or HT29 cells with the noninvasive mxiD or BS176 strains was followed by strong transcriptional
induction of HBD2, HBD3, LL37, CCL20, and CCR6 genes, over their basal levels that could be observed as early as 1 h 30 min after infection, whereas the HBD1 gene remained expressed at its basal level of expression throughout the experiments (unpublished data).

In our experimental conditions, the invasive wild-type strain M90T was unable to significantly invade polarized and differentiated cell monolayers, as assessed by gentamicin assays (Fig. 1 C), which is in agreement with data obtained in apically infected polarized Caco-2 cells (14). However, infection experiments with strain M90T showed differential transcriptional expression of the HBD3 and CCR6 genes in both cell types, and for the LL37 gene in TC7 cells only, compared with infections with the noninvasive mxiD and BS176 strains (Fig. 1, A and B). At 4 h 30 min after infection, transcription of these genes in infected cells was induced to a lesser extent by the wild-type strain M90T compared with the mxiD and BS176 strains. In TC7 cells, the HBD3 gene expression was induced only 4-fold by M90T compared with >110-fold by BS176 (Fig. 1 A). Conversely, expression of HBD1, HBD2, and CCL20 genes was found to be similar for all infections with the two cell lines (Fig. 1, A and B). As a control, cellular viability was the same for monolayers infected with the wild-type S. flexneri strain and its derivatives, as assessed by lactate dehydrogenase assays (Fig. 1 D). Collectively, these results indicate that wild-type S. flexneri impairs expression of specific innate immunity genes by injecting virulence factors into epithelial cells through its TTSS, thereby affecting the host immune response.

The S. flexneri MxiE regulon includes >10 virulence proteins delivered through the TTSS upon contact of the bacteria with cells. Among them, the OspF and OspG effectors have been shown to target the host NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways (20, 21, 27, 28). Expression of the genes encoding these proteins is dramatically affected in a mxiE mutant, therefore preventing their synthesis and injection into cells upon infection (19). However, the mxiE mutant still has a functional TTSS for injection of the MxiE-independent effectors, allowing an efficient invasion of nonpolarized epithelial cells, comparable to the M90T wild-type strain (19, 47). To determine whether the pool of MxiE–dependent effectors was involved in the host innate immunity gene’s down-regulation, we carried out infection experiments with the invasive mxiE mutant. Like observed for the wild-type strain M90T, the mxiE mutant was unable to significantly invade polarized and differentiated TC7 or HT29 cells (Fig. 1 C). However, in contrast to M90T, the mxiE mutant was unable to modulate transcription of HBD3, LL37, and CCR6 genes in infected cell monolayers (Fig. 1, A and B). Transcription of these genes, as well as of the other

Figure 1. Virulent S. flexneri modulates transcription of innate immunity genes in polarized colonic epithelial cells. (A and B) Expression of β-defensins HBD1, HBD2, and HBD3, cathelicidin LL37, chemokine CCL20, and chemokine receptor CCR6 encoding genes was determined in TC7 (A) or HT29 (B) cell monolayers 4 h 30 min after infection (3 h after gentamicin addition). After RNAs extractions and RT reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes in each condition, using the comparative Ct method. Results are presented on a logarithmic scale as the ratio of gene expression in infected cells compared with noninfected cells. Black bars, wild-type strain M90T; gray bars, plasmid-cured BS176 strain; white bars, mxiD mutant strain; striped bars, mxiE mutant strain. *, P < 0.01 for infections with the wild-type strain compared with the other strains. (C) S. flexneri is unable to significantly invade polarized and differentiated TC7 or HT29 epithelial cells. Gentamicin assays were performed in 6-well plates (1.5 × 10⁶ cells/well), at a MOI of 100, in conditions similar to those used for infection experiments (time course: 1 h 30 min). Values represent the percentage of intracellular CFU. Black bars, TC7 cells; gray bars, HT29 cells. (D) Cellular viability is the same for TC7 or HT29 cell monolayers infected with the wild-type S. flexneri strain and its derivatives. Lactate dehydrogenase assays were performed in 6-well plates (1.5 × 10⁶ cells/well), at a MOI of 100, in conditions similar to those used for infection experiments (time course: 1 h 30 min). Values represent the percentage of cell death. Black bars, TC7 cells; gray bars, HT29 cells. n = 3 independent biological experiments. Error bars represent the SD.
genes studied, was similar in cells upon mxiE, mxiD, or BS176 infection (Fig. 1, A and B). In addition, infection experiments performed with mutant strains defective for the ospF or ospG genes revealed that the encoded effectors repressed antimicrobial factor genes transcription (unpublished data). However, the extent of repression was inferior to the one observed with the whole set of effector proteins delivered by the M90T strain, suggesting additive synergistic action of these effectors to get full repression of the antimicrobial peptide genes expression. Thus, the pool of MxiE–dependent effectors injected in cells upon infection is responsible for the observed manipulation of the host immune response through transcriptional dampening.

Expression of several genes of the innate immune response is known to be up-regulated in intestinal epithelial cells, by treatment with proinflammatory molecules such as IL-1β, TNF-α, or IFN-γ (4). To determine whether S. flexneri was still able to modulate transcription of innate immunity genes in cells induced to undergo an inflammatory response pattern, TC7 cell monolayers were stimulated by IL-1β for 2 h, the IL-1β–containing medium was removed, and S. flexneri infections were performed. IL-1β treatment was followed by an induction of the HBD2, HBD3, and CCL20 genes transcription in noninfected cells (Fig. 2). In the same conditions, expression of the HBD1 gene remained unchanged, whereas a weak down-regulation was observed for CCR6 gene expression (Fig. 2). Interestingly, the wild-type S. flexneri strain M90T conserved its capacity to actively repress HBD3 gene expression in IL-1β–stimulated cells (Fig. 2). As expected, the mxiE mutant strain, as well as the mxiD and BS176 strains, were still unable to modulate HBD3 gene induction in IL-1β–treated cells (Fig. 2). Similarly, wild-type bacteria repressed CCR6 gene transcription in stimulated cells, whereas mxiD and mxiE mutants failed to dampen its expression in similar conditions (Fig. 2). Collectively, these in vitro results indicate that virulent S. flexneri manipulates the host innate immune response through injection of the MxiE–dependent effectors.

Figure 2. S. flexneri modulates transcription of innate immunity genes in cells expressing an inflammatory response pattern. Expression of the β-defensins HBD1, HBD2, and HBD3, chemokine CCL20, and chemokine receptor CCR6 encoding genes was determined at 4 h 30 min after infection (3 h after gentamicin addition) in TC7 cells that were prestimulated for 2 h by IL-1β. After RNA extractions and reverse transcription reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes in each condition, using the comparative Ct method. Results are presented on a logarithmic scale as the ratio of gene expression in stimulated and infected cells compared with nonstimulated and noninfected cells. Light gray bars, noninfected; black bars, wild-type strain M90T; gray bars, plasmid-cured BS176 strain; white bars, mxiD mutant strain; striped bars, mxiE mutant strain. n = 3 independent biological experiments. Error bars represent the SD. *, P < 0.01 for infections with the wild-type strain compared with the other strains.

Figure 3. Antimicrobial factors whose transcription is repressed by S. flexneri are those affecting highest bactericidal activity against the pathogen. (A) Bactericidal activities of synthetic β-defensins hBD-1, hBD-2, and hBD-3, cathelicidin LL37, or recombinant chemokine CCL20 were determined on S. flexneri exponential cultures growing in DME medium for 2 h at 37°C. hBD-1, hBD-2, and hBD-3, LL37 were used at 50 μg/ml, whereas CCL20 was used at 5 μg/ml final concentration. Values expressed in arbitrary fluorescent units (afu) represent the mean fluorescence of the bacterial population analyzed. Black bars, wild-type strain M90T; gray bars, plasmid-cured BS176 strain; white bars, mxiD mutant strain; striped bars, mxiE mutant strain. *, P < 0.01 for the fluorescence obtained with the BS176 strain compared with the other strains. (B) L monocytogenes and L innocua were used as positive controls for antimicrobial activity of these factors inefficient on S. flexneri in our experimental conditions. Black bars, L monocytogenes; gray bars, L innocua. n = 3 independent experiments. Error bars represent the SD.
even in cells already expressing an inflammatory pattern, a situation that is likely to prevail in the dynamics of interactions between luminal bacteria and the epithelial lining in the course of an infection.

**Antimicrobial factors whose transcription is repressed by *S. flexneri* are those affecting the highest bactericidal activity against the pathogen**

To measure the antimicrobial activity of innate immune factors such as β-defensins hBD-1, hBD-2, and hBD-3, cathelicidin LL37, and chemokine CCL20 on *S. flexneri* viability, we determined the in vitro antimicrobial activity of each of these peptides against the pathogen, using synthetic or recombinant molecules. Typically, the conditions used for demonstrating activity of antimicrobial peptides include buffers of low ionic strength and neutral pH that allow for optimal killing because the activity of most antimicrobial factors is antagonized by high salt concentrations, the presence of divalent cations, and serum components (4). For this purpose, the wild-type strain M90T and its derivatives mxiD, mxiE, and BS176, were sampled in the exponential phase, resuspended, and cultured for 2 h in 1:2 diluted DME culture medium (salts 0.08 M, pH 7.2; no serum) containing the relevant antimicrobial peptide. The DiBAC$_{4}$ fluorescent reagent that is able to cross-depolarize bacterial membranes was added to cultures, followed by FACS analysis of bacterial fluorescence (mean fluorescence) (29).

In these conditions, β-defensins hBD-1 and -2 were inactive on the *S. flexneri* wild-type strain and its derivatives, as assessed by the weak fluorescence values obtained (Fig. 3A). Conversely, the β-defensin hBD-3 exhibited strong bactericidal activity on the pathogen at a similar concentration (Fig. 3A). Interestingly, the plasmid-cured BS176 strain was twofold more sensitive to hBD-3 than the other strains, as confirmed by CFU assays (unpublished data), suggesting that proteins encoded by the virulence plasmid achieve higher bacterial resistance to this antimicrobial factor. To explore the possibility of plasmid-encoded proteolytic degradation of hBD-3, we...

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**Figure 4. *S. flexneri* manipulates expression of genes from the innate immune response in human intestinal xenografts.** (A) Human tissues were infected for 2 h by direct intraluminal inoculation with 5 × 10$^7$ bacteria. Total RNA was extracted from a section of each infected graft, cRNA were synthesized and hybridized on the Affymetrix U133A GeneChip Human Array. Gene expression profiles from two experiments corresponding to the noninfected condition (NI), BS176 infection (BS176), M90T infection (M90T), and mxiE infection (mxiE), are shown. Hierarchical clustering of immunity genes was performed using dChip software with Euclidian distance and average as a linkage method. Before clustering, expression values for one gene across all samples were standardized to produce a mean of zero. Increased or decreased values were then ranged compared with that mean. Red and blue colors represent expression that is higher or lower than the mean value, respectively. The key for intensity of expression is indicated under the bar. (B) Validation/determination by qRT-PCR of β-defensins HBD1, HBD2, and HBD3, cathelicidin LL37, chemokine CCL20, and chemokine receptor CCR6 genes expression in infected tissues used for GeneChip experiments. After reverse transcription reactions, qRT-PCR was performed on each sample with specific primers to determine the relative expression of genes in each condition using the comparative Ct method. Results are presented on a logarithmic scale as the ratio of gene expression in infected tissues compared with noninfected tissues. Black bars, wild-type strain M90T; gray bars, plasmid-cured BS176 strain; striped bars, mxiE mutant strain. $n = 2$ independent biological replicates per condition.
tested the activity of the defensin in the presence of *S. flexneri* mutants defective for the expression of the surface/secreted proteases SepA or SopA that are encoded by the virulence plasmid, and the *sepA*-BS176–complemented strain (18, 30). No change in bacterial sensitivity was observed with any of the mutant strains, suggesting that *S. flexneri* is unlikely to proteolytically degrade the antimicrobial factors (unpublished data). Finally, among the antimicrobial factors tested, cathelicidin LL37 and chemokine CCL20 exhibited an intermediary antibacterial action on *S. flexneri* compared with hBD-1–2 and hBD-3 (Fig. 3A). As a control of our experimental conditions, *Listeria monocytogenes* and *Listeria innocua* were tested and found to be sensitive to all the antimicrobial peptides tested, except for cathelicidin LL37, at equivalent concentrations (Fig. 3B). Collectively, these results show that among the molecules tested in vitro, the most active antimicrobial factors for *S. flexneri* are those whose expression is repressed by the virulent bacterium at the transcriptional level.

*S. flexneri* regulates expression of innate immunity genes in vivo

To confirm in vivo that virulent *S. flexneri* down-regulates expression of antimicrobial factors, we performed a transcriptomic analysis of xenotransplanted human intestinal segments after infection for 2 h by the wild-type strain M90T, the *mxiE* mutant, and the avirulent strain BS176. Xenografts infected with bacterial medium alone were used as a reference. Xenotransplants exhibit a chimerical structure, combining essentially human intestinal epithelium and mouse vascular and immune cells. Infection of these tissues for short time periods allowed for the study of gene transcription in human epithelial cell, thanks to the use of human–specific arrays. Two independent array hybridization experiments were performed for each condition, and the hierarchical clustering of modulated gene expression was determined. Among them, we specifically focused our attention on genes encoding chemokines, cytokines, and antimicrobial peptides (Fig. 4A). Validation of data were performed on a set of genes, including HBD2 and HBD3, whose probe sets were absent from the used arrays, using qRT–PCR (Fig. 4B). From this gene cluster, we identified a total of 46 genes whose expression was significantly modulated by *S. flexneri* in infected intestinal xenotransplants.

Interestingly, 11 genes were less transcribed upon infection with the wild-type strain M90T compared with the invasive *mxiE* mutant or the noninvasive BS176 strain (Fig. 4, A and B). These targets of the MxiE–dependent effectors included genes encoding chemokines and chemokine receptors, such as CCL3, CCL4, CCL20, CCL25, and CCRL2. Mainly produced by epithelial cells, these proteins are involved in the recruitment and activation of inflammatory and immune effectors cells such as monocytes, DCs, and T cells (31). Expression of genes encoding cytokines IL-7 and -18 and cytokine receptors IL-13RA1 and -20RA was also less expressed in human tissues infected by the M90T strain compared with the *mxiE* mutant. IL-7 is produced locally by intestinal epithelial and goblet cells, and may serve as a regulatory factor for intestinal mucosal lymphocytes, whereas IL-18 induces IFN-γ production by T cells. Finally, among down-regulated genes, as measured by qRT–PCR, expression of β-defensins HBD1 and HBD3 was strongly reduced in xenotransplants infected with wild-type bacteria compared with *mxiE* mutants; ratios were 0.09:1.09 for HBD1 and 0.1:0.82 for HBD3, respectively (Fig. 4, A and B).

In addition to down-regulating the expression of several genes encoding chemokines, cytokines, and antimicrobial peptides, transcriptional analysis indicated that MxiE–dependent bacterial effectors up-regulate the expression of the SOCS2 gene (Fig. 4A). Interestingly, members of this family are cytokine-inducible negative regulators of cytokine signaling pathways whose primary function is to attenuate JAK–STAT

**Figure 5.** *S. flexneri* blocks hBD-1, hBD-3, and CCL20 expression in human intestinal xenografts. Anti–human hBD-1 (A and B), anti–human hBD-3 (C and D), and anti–human CCL20 (E and F) immunostainings of human intestinal xenograft sections after 2 h of infection with the wild-type *S. flexneri* strain M90T (A, C, and E) or the *mxiE* mutant (B, D, and F). Arrows indicate the poor luminal release of hBD-1 (A), hBD-3 (C), and CCL20 (E) by epithelial cells, typically observed in tissues infected by wild-type bacteria. Arrowheads highlight areas of extensive release of hBD-1 (B), hBD-3 (D), and CCL20 (F) by epithelial cells, typically found in *mxiE*-infected tissues. *n* = 4 independent biological replicates per condition. Bars, 10 μm.
signaling. In turn, a potential role of the JAK–STAT signaling pathway in down-regulating expression of HBD1 and HBD3 has been suggested (32). Collectively, these in vivo results show that *S. flexneri* is able to manipulate the host innate immune response through its MxiE-dependent effectors by up- or down-regulating expression of crucial innate immunity genes.

*S. flexneri* blocks antimicrobial factors expression in vivo

Our study so far has shown that MxiE-dependent *S. flexneri* effectors modulate expression of a subset of genes encoding molecules involved in the host innate immune response. Therefore, we investigated the in vivo consequences of this strategy in intestinal tissues infected by the pathogen. For this purpose, xenotransplants were infected for 2 h with the wild-type strain M90T, the mxiE mutant, the noninvasive mxiD mutant harboring an inactive TTSS, or injected with bacterial culture medium alone as control. Immunolabelings were performed on infected tissues with antisera to human β-defensins hBD-1, hBD-3, and chemokine CCL20. Noninfected tissues revealed weak staining for hBD-1 and no labeling for hBD-3 and CCL20, which is in agreement with the respective constitutive and inducible production of these molecules by epithelial cells in vivo (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20071698/DC1). Interestingly, in tissues infected by the wild-type strain M90T, hBD-1 and -3 staining exhibited a weak labeling after the villi contours, suggesting poor synthesis and release of these molecules in the lumen (Fig. 5, A and C). Conversely, in mxiE mutant-infected tissues, massive luminal release of these antimicrobial peptides was observed from epithelial cells exposed to the colonizing bacteria, imprinting the portion of the mucus layer proximal to the epithelial surface (Fig. 5, B and D). As expected for their profile, β-defensin hBD-2 immunostainings were similar in human xenografts infected by wild-type and mxiE mutant bacteria (unpublished data). In agreement with the transcriptional data from xenografts, CCL20 labeling showed strong production of this chemokine by enterocytes along villi with luminal efflux in the transplants infected by the mxiE strain, whereas only a week signal was detected in tissues infected by the M90T strain (Fig. 5, E and F). Other defensin- and chemokine-staining experiments indicated a similar staining pattern for mxiD− compared with mxiE-infected xenografts (Fig. S1, B, D, and F). Collectively, these experiments indicate that MxiE-dependent *S. flexneri* effectors affect the production of hBD-1, hBD-3, and CCL20 molecules exhibiting antimicrobial activities, thereby weakening the antimicrobial defense barrier at infected mucosal surfaces.

Blocking of antimicrobial factor expression correlates with deeper progression of *S. flexneri* toward intestinal crypts

Such epithelial cell manipulation for antimicrobial factor expression may represent a critical mechanism allowing *S. flexneri* to more deeply colonize and invade the mucosa. At 2 h after infection, the presence of the M90T wild-type strain and the mxiE mutant in the lamina propria of infected xenotransplants was observed only in few occasions (unpublished data). However, the strong epithelial expression of antimicrobial factors observed at the same time indicates the establishment of a mucosal inflammatory response (Fig. 5 and Fig. S1). This situation prompted us to study how the epithelial inflammatory response affected the behavior of bacteria at the mucosal surface, especially their ability to progress toward crypts through intervilli spaces. For this purpose, we performed a qualitative and quantitative analysis of the bacterial distribution from the top of the villi to the crypts of xenotransplants infected for 2 h with the wild-type strain M90T or with the mxiE and mxiD mutants. Qualitatively, *S. flexneri* 5a LPS immunostainings revealed that wild-type bacteria were distributed more diffusely in the mucus layer than mxiE and mxiD mutants, whose localization remained close to the top of the villi, essentially trapped in the luminal mucus (Fig. 6, A–C). In addition, mxiE and mxiD mutants were barely seen localized in luminal intervilli spaces or deep near the crypts, compared with wild-type bacteria that affected a much deeper diffusion pattern (Fig. 6, A–C). Quantitatively, the amount of M90T bacteria...
observed in the lower third of the villi, including the crypt areas, was close to the half of the one determined at the upper third (Fig. 6 D). Collectively, these results highlight the correlation existing between the ability of virulent S. flexneri to block antimicrobial factors expression, and their capacity to progress deeply and massively toward intestinal crypts, at the early time point of infection.

S. flexneri compromises recruitment of DCs to the lamina propria of infected tissues
The β-defensins hBD-1 and -3, and the chemokine CCL20, are immune effectors known to have both antimicrobial properties and chemotactic activities. Our study so far has shown that the blocking of their expression at the mucosal surface by S. flexneri was coupled to deeper progression of bacteria toward crypts. To correlate these findings to mouse immune cell trafficking, additional immunostaining experiments were performed using a monoclonal antibody to mouse CD11c, one of the main antigens present on the surface of DCs. CD11c staining of mxiE-infected tissues revealed massive recruitment of DCs into the lamina propria and submucosal regions; they are probably attracted by the gradient of chemotactic molecules, such as CCL20, emanating from epithelial cells (Fig. 7 B). Similar observations were obtained in mxiD-infected tissues (Fig. 7 D). In contrast, M90T-infected xenotransplants showed a very different pattern characterized by a restricted presence of DCs in submucosal areas, as well as a poor migration into the lamina propria with much less staining than observed in noninfected tissues for resident DCs (Fig. 7, A and C). Collectively, these experiments highlight the existence of a dedicated MxiE-dependent system allowing S. flexneri to suppress expression of immune effectors, leading to compromised recruitment of DCs to the lamina propria of infected tissues.

DISCUSSION
The human intestinal epithelium achieves a barrier function between the host’s “milieu intérieur” and the outside environment, particularly the gut microbiota. This barrier protects against invasion and systemic dissemination of both commensal and pathogenic microorganisms. The epithelium can be seen as comprising three barriers in one: a physical barrier, an innate immune barrier, and an adaptive immune barrier (33). Accessibility to the apical surface of epithelial cells and rupture of the physical barrier are among the main properties that distinguish pathogens from commensals. Among the numerous factors that participate in establishment and maintenance of epithelial homeostasis, antimicrobial factors that are ubiquitously expressed by epithelial cells throughout the gastrointestinal tract play an important regulatory role in controlling the resident and transient bacterial populations (34). Moreover, epithelial cells and DCs act as sentinels at mucosal surfaces and secrete chemokines that recruit, activate, and regulate inflammatory infiltrates, including polymorphonuclear leukocytes transmigrating through the epithelium, resident macrophages, recruited monocytes, and a variety of lymphocytes. These effectors participate in the innate response to commensals under steady-state conditions, a situation of tolerance that commensals themselves actively maintain (35). In addition to maintaining efficient physical and innate immune barrier functions, the intestinal epithelium also takes an active part in the induction of adaptive immune surveillance at the mucosal surface. After sampling luminal bacteria, DCs migrate to mesenteric lymph nodes and initiate an adaptive immune response directing specific IgA production in the lamina propria (36). Mucosal defense mechanisms also encompass cellular factors, including B and T cells, and lymphocytes unique to the gut-associated lymphoid tissues, such as lamina propria lymphocytes (37).

However, homeostasis of the epithelial barrier can be subverted by crosstalks between enteric bacterial pathogens and mucosal tissues. The genetic make up of bacterial enteric pathogens can therefore be seen in the perspective of a co-evolutionary process that has led to the presence of genes and pathogenicity island encoding effectors able to disrupt this homeostatic process and to resist to its essentially inflammatory consequences. Because antimicrobial peptides appear to be primary effectors throughout evolution to fight microbes and represent in the vast majority of species, particularly in insects, the first line of defense in the absence of an adaptive immune response (38), it would not be surprising if the genome of pathogens such as S. flexneri had evolved to respond to this vital constraint. How this is achieved is just being unraveled.
Epithelial cells mediate and orchestrate this dialog mainly through activation of proinflammatory pathways, particularly the NF-κB– and activating protein 1– (AP-1) signaling pathways (39). To gain access to the epithelial surface, to colonize it, and to then possibly disrupt this barrier and invade the mucosa, pathogens have evolved strategies to overcome the development of antimicrobial immune responses. Pathogenic bacteria, such as *S. flexneri*, *Salmonella*, or *Verrinia*, have elaborated strategies targeting specific steps of the NF-κB and MAPK pathways that allow them to interfere with the transcription of immune response genes from epithelial cells (20, 39).

Rectal biopsies from patients with bacillary dysentery revealed that expression of the antibacterial peptides HBD1 and LL37 was reduced (22, 23). This was the first indication that *S. flexneri* might abrogate expression of some key antimicrobial peptides as part of a possibly more global strategy of manipulating the mucosal innate immune response. Thus, this observation needed to be confirmed and analyzed in light of accumulating data on *S. flexneri* pathogenesis, particularly the evidence that the TTSS of *S. flexneri* allows injection of protein effectors that have the capacity to target specific steps of activation of key innate response pathways, such as the NF-κB pathway into epithelial cells. OsPF, which is one of the plasmid-encoded MxiE-regulated proteins, is a kinase that binds a limited set of ubiquitinated E2 enzymes, particularly those involved in the degradation of IκB, thereby blocking their function and preventing translocation of the NF-κB complex into the nucleus (21). On the other hand, the OsPF effector, which is a phosphothreonine lyase that reaches the host cell nucleus, dephosphorylates the MAPK Erk1/2 and p38, thereby leading to the dephosphorylation of histone H3. Subsequent epigenetic modifications impair the accessibility of the NF-κB complex on a series of promoters (20, 28). The osPF and osPG genes are essentially under the control of MxiE, the master regulator of the regulon encoding >10 plasmid-borne genes (19). Their transcription is triggered by activation of the TTSS; thus, as soon as bacteria establish contact with target cells, these proteins are expressed and secreted into cells that, at the same time, undergo strong proinflammatory signaling via the TLR and nucleotide oligomerization domain pathways. This system offers the opportunity to bacteria to suppress the expression of genes whose promoters contain NF-κB and AP-1 motifs, as well as possibly other regulatory sequences.

Antimicrobial peptide–encoding genes have mosaic promoters characterized by the presence of NF-κB, AP-1, and others regulatory boxes. Given our current understanding of signaling pathways targeted by the MxiE-regulated effectors, these genes are therefore excellent candidates to be regulated by the set of effectors under the mxiE regulon. This is the case for the β-defensin HBD2 whose expression has been shown to be mediated through NF-κB, sometimes assisted by AP-1, MAPK, and protein kinase C (40, 41). A potential role of the JAK2–STAT signaling pathway has been suggested in the control of HBD1 and HBD3 expression (32). Indeed, the HBD3 gene promoter contains transcriptional binding motifs for AP-1, IFN-γ interferon response elements, and NF-IL-6 response elements. Moreover, the inhibition of HBD3 gene transcription observed with both the NF-κB inhibitor PDTC and the p38 inhibitor suggests the possibility of the existence of NF-κB binding motifs (42). As suggested earlier, one could even hypothesize that the selective pressure for acquisition of the diversity of Osp and possibly IpH effectors, the latter being a new class of E3 ubiquitin ligases with no known substrate as yet (43), has been partly driven by the need to transcriptionally suppress expression of defense genes whose activation and regulation is complex and multifactorial.

Antimicrobial peptides exhibit a strong antibacterial activity toward Gram-negative and -positive bacteria, as observed for hBD-3 on *S. flexneri* and *Listeria*, as well as an ability to act as chemoattractants for immune cells (4). With such properties, it seems logical that pathogens have developed mechanisms to resist these immune effectors. The main strategies for pathogenic bacteria to thwart cationic peptides are to die and act as decoys, to degrade them, to block their action, or to suppress their expression, particularly at the transcriptional level (44). The latter option appears to be the strategy that *S. flexneri* has evolved, along with other pathogens such as *Salmonella* and *Neisseria*, although the mechanisms used by these two pathogens to interfere with the host signaling pathways are so far uncharacterized (24, 25). Finding the *S. flexneri* effectors and the molecular mechanisms that dampen the transcription of antimicrobial peptides will lead to a better understanding of the complex circuits that induce and regulate their expression, and will provide another example of the contribution of bacterial virulence factors to decipher basic physiological mechanisms (45).

Exposure of the intestine to microbes maintains steady-state “physiological inflammation” at mucosal surfaces, a situation preventing tissue invasion by commensal microorganisms. In this scheme, DCs orchestrate the innate and the adaptive immune responses, playing a central role in properly interpreting signals that maintain tolerance versus those that should trigger a neutralizing adaptive immune response against pathogens. As such, pathogenic bacteria have multiple opportunities to subvert the immune response at the level of DCs. In this study, *S. flexneri* was found to down-regulate innate immunity genes such as CCL20, the main DC chemoattractant, leading to compromised recruitment of DCs to the lamina propria of infected tissues. By blocking the recruitment of DCs to the site of infection, *S. flexneri* potentially interferes with the tolerogenic process, and thus uncontrolled inflammation that is known to facilitate disruption of the epithelial barrier and facilitate further mucosal invasion (17). An inhibition of DC recruitment may also strongly interfere with the critical step of transition between the innate and adaptive response, which will decide upon the switch toward a Th1, Th2, or Th17 response, the development of this response, and the triggering of immunological memories. These issues are essential to bear in mind for the development of a rationally attenuated live oral vaccine candidate against *S. flexneri*. The difficulty of the equation now appears to maintain a strong protection against killing by epithelial defenses to achieve
proper colonization, to control the intensity of the inflammatory response to achieve optimal tolerance while disconnecting the mechanisms of immunosuppression to achieve maximum immunogenicity and memory.

MATERIALS AND METHODS

Bacterial strains and culture conditions. S. flexneri serotype 5a strains were isolated on Congo red agar plates. The invasive wild-type strain M90T (46) and its isogenic derivatives, the invasive mxiE mutant (47) (impaired for the MxiE transcriptional activator regulating expression of several virulence plasmid-encoded effectors), the noninvasive mxiD mutant (48) (impaired for the MxiD protein, a component of the TTSS required for its functionality), and the noninvasive plasmid-cured BS176 strain (46) were used. For infection experiments, strains were cultured overnight in BTCS medium (Difco) at 37°C with shaking. For human intestinal xenograft infections, overnight cultures were directly used. For polarized and differentiated monolayer cell infections, after fresh dilutions, subcultures were performed for 2 h to reach the exponential phase, spun gently, and used after resuspension of bacteria in DME tissue culture medium (Invitrogen).

Polarized and differentiated cell monolayer infections. TC7 (49) and HT29 human epithelial cells derived from colonic carcinoma were cultured in 6-well plates (1.5 × 10⁵ cells/well) with DME (Invitrogen) supplemented with 10% FCS (Invitrogen), 1% nonessential amino acids (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) in 10% CO₂. Grafts were allowed to develop for 10 wk before use, were placed into the subscapular region of 6–8-wk-old SCID mice, as previously described (51). 10 μg of cRNA were fragmented and hybridized with 5 × 10⁷ K50 -derived cRNA (Affymetrix). Analysis of DiBAC₄-fluorescent bacteria was performed before acquisition. Analysis of K50 -containing medium was removed and infections were performed. To facilitate S. flexneri infection, the cell culture plates were spun gently for 10 min at 1,000 rpm at room temperature, and then stored at 37°C. After 1 h 30 min of infection, 50 μg/ml gentamicin (Invitrogen) was added, cells were incubated for the appropriate length of time, washed with PBS, and lysed for RNA isolations. Gentamicin assays (14) and lactate dehydrogenase assays (CytoTox; Promega) were performed on TC7 or HT29 cell monolayers under similar conditions of infection.

Human intestinal xenograft infections. Human intestinal xenografts were placed into the subcapsular region of 6–8-wk-old SCID mice, as previously described (50). Grafts were allowed to develop for 10 wk before use, and then human tissues on mice were infected by direct intraluminal inoculation with 5 × 10⁷ S. flexneri bacteria. A group was mock infected by inoculation with media alone, as a control. At 2 h after infection, animals were killed, and grafts were removed and frozen for Trizol RNA isolation (Invitrogen). To obtain polarized and differentiated cell monolayers, cells were grown for 3 wk to reach full differentiation and polarization. Culture medium was changed three times per week. Infections were performed in triplicate in DME without serum at a multiplicity of infection (MOI) of 100. When specified, cells were stimulated for 2 h with 20 ng/ml IL-1β (R&D Systems), after which IL-1β-containing medium was removed and infections were performed. To facilitate S. flexneri infection, the cell culture plates were spun gently for 10 min at 1,000 rpm at room temperature, and then stored at 37°C. After 1 h 30 min of infection, 50 μg/ml gentamicin (Invitrogen) was added, cells were incubated for the appropriate length of time, washed with PBS, and lysed for RNA isolations. Gentamicin assays (14) and lactate dehydrogenase assays (CytoTox; Promega) were performed on TC7 or HT29 cell monolayers under similar conditions of infection.

RNA isolation and quantitative real-time PCR. RNAs were isolated using the RNeasy Mini kit. RT-PCR reactions were performed on 4-μg RNA samples using the SuperScript II reverse transcription (Invitrogen) and the oligo(dT)15 primers (Promega) as recommended by the suppliers. Gene-specific primers were purchased from Proligo (Sigma-Aldrich): HB1D1, 5′-ACGGTGTTAATCTTTCACAGG-3′ and 5′-ATATAGACACATGTTCCCTC-3′; HB2D, 5′-GCCATGAGGTAAACAGGATCG-3′; HB3D, 5′-GTGTTGTCCAGTACATGGTAGAG-3′ and 5′-TATACAGTCCGGCACCCAGC-3′; LL37, 5′-CGGAAATCTAAGAAGATTGG-3′ and 5′-TACGGCCACCATGACTAGCTCT-3′; CCL20, 5′-GACATACGCAAGAACAAGAAGG-3′ and 5′-TAATTGGCACAGGTCCAGTGAGG-3′; CCR6, 5′-CAGCCTCATCCTCCAGCCTCAAC-3′ and 5′-CTCCGGAGACGTCTGATTCTTG-3′. qRT-PCRs were carried out in a 20-μl volume containing 8 μl of cDNA (diluted at 1/100), specific primers (0.2 μM each), and 10 μl of Power SYBR Green mix (Applied Biosystems). Reactions were run on an ABI 7900HT instrument (Applied Biosystems) with conditions of the recommended universal thermal cycling parameters. Each reaction was run in duplicate. Relative quantification of gene expression was performed using the comparative Ct method. Results were normalized using the human β-2-microglobulin (B2M) housekeeping gene.

Immunohistochemical experiments. Cryostat sections (7 μm) were prepared from human intestinal xenografts conserved in OCT. Sections were rehydrated, blocked for 30 min in PBS with 1% BSA, and immunostained with the following antibodies: polyclonal antibody to S. flexneri 5a LPS, polyclonal antibody to human hBD-1 (FL-68; Santa Cruz Biotechnology), polyclonal antibody to human hBD-3 (FL-67; Santa Cruz Biotechnology), polyclonal antibody to human CCL20 (AF360; R&D Systems), and monoclonal biotinylated antibody to mouse CD11c (HL3; BD Biosciences). Slides were washed twice in PBS before addition of the appropriate peroxidase-conjugated secondary antibody. Detection was accomplished by addition of the liquid DAB Substrate-Chromogen System (Dako).

Antimicrobial assays. Antimicrobial activities of the synthetic human β-defensins hBD-1, hBD-2, hBD-3 (PeptaNova), cathelicidin LL37 (Phoenix Pharmaceuticals), and the recombinant chemokine CCL20 (PeproTech) were tested on the different S. flexneri strains, as previously described (29). In brief, fresh dilutions of overnight S. flexneri cultures were subcultured for 2 h, bacteria were spun gently, resuspended in DME medium (Invitrogen) containing 5 μg/ml (CCL20) or 50 μg/ml (hBD1-3 and LL37) of the relevant peptide, and incubated for 2 h at 37°C with intermittent shaking, in a 100-μl final volume. Because the activity of most antimicrobial factors is antagonized by high salt concentrations, DME medium was diluted to 1:2 for these experiments (4). Bacteria were incubated for 10 min at room temperature with 10 μg/ml bis-(1,3-dibutylbarbituric acid)-thimethine oxonol fluorescent molecule (DiBAC₄; Invitrogen), which crosses depolarized bacterial membrane only, before acquisition. Analysis of DiBAC₄-fluorescent bacteria was performed with a FACSCalibur apparatus (BD Biosciences) using the CellQuest Pro software. The mean fluorescence is presented in arbitrary fluorescent units.
Bacterial counts in infected xenograft tissues. The progression of S. flexneri toward intestinal crypts of infected xenograft tissues was evaluated by determining and comparing the amount of bacteria from the top of the villi to the crypts of the mucosa. In brief, sections from xenograft tissues infected by the wild-type S. flexneri strain M90T, and the mexD and mexE mutants, were immunostained with a polyclonal antibody to S. flexneri 5a LPS, and used to count the bacteria localized at the top of the villi (upper third), in the luminal intervilli spaces (intermediate third), and in the crypts (lower third) of the mucosal tissues. We systematically analyzed a representative set of 10 villi per section, and 3 sections were read for 4 independent infected xenograft tissues. Data are presented as a percentage of the amount of bacteria. The 100% is defined by the amount of M90T bacteria determined at the upper third of the villi.

Online supplemental material. Fig. S1 shows that noninvasive S. flexneri are unable to modulate hBD-1, hBD-3, and CCL20 expression in human intestinal xenografts. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20071698/DC1.

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