Neutrophil antimicrobial proteins enhance *Shigella flexneri* adhesion and invasion

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**Summary**

*Shigella flexneri* is an enteric pathogen that causes massive inflammation and destruction of the human intestinal epithelium. Neutrophils are the first cells of the innate immune system recruited to the site of infection. These cells can attack microbes by phagocytosis, Neutrophil Extracellular Trap (NET) formation and degranulation. Here, we investigated how neutrophil degranulation affects virulence and show that exposure of *Shigella* to granular proteins enhances infection of epithelial cells. During this process, cationic granular proteins bind to the *Shigella* surface causing increased adhesion which ultimately leads to hyperinvasion. This effect is mediated by changes in the surface charge, since a lipopolysaccharide (LPS) mutant with a negative surface shows enhanced hyperinvasion compared with wild-type *Shigella*. We propose that *Shigella* evolved to use host defence molecules to enhance its virulence and subvert the innate immune system.

**Introduction**

*Shigella flexneri* is a Gram-negative enteropathogenic bacterium that causes severe dysentery in humans.

Shigellosis is responsible for 91 million infections and 414 000 deaths in Asia and over 8 million infections in Africa per year (Anonymous, 2005). This infection is highly contagious; only 10–100 bacteria are sufficient to cause disease in healthy individuals (Dupont et al., 1989).

*Shigella* infects the mucosa of the colon where it breaches the integrity of the epithelial barrier and leads to severe inflammation. The current model of pathogenesis is that *Shigella* traverses from the lumen of the intestine to the submucosa through M-cells. There, *Shigella* actively invades epithelial cells and then spreads to other cells in the epithelium. Indeed, this bacterial pathogen can only invade host cells through the basolateral membrane. This invasion process requires virulence factors secreted by the *Shigella* type three secretion system (TTSS). The genes encoding the virulence factors and the TTSS are located on the *Shigella* large virulence plasmid (Baudry et al., 1987). Invasion of epithelial cells leads to activation of the NF-κB pathway and release of IL-8 (Philpott et al., 2000). IL-8, in turn, attracts neutrophils very early to the site of infection (Sansonetti et al., 1999) where they are activated (Baggiolini et al., 1989). Neutrophils play a paradoxical role in shigellosis, since they are required not only for the full onset of epithelial destruction but also for clearance of the infection (Perdomo et al., 1994a; Mandic-Mulec et al., 1997).

Lipopolysaccharide (LPS), a major bacterial virulence determinant (Trent et al., 2006), consists of Lipid A, a core region and the O-antigen. The Lipid A and the core region are negatively charged because they contain 3-deoxy-D-manno-octulosonic acid (KDO) and phosphorylated heptose. The O-antigen consists of multiple repeats of uncharged sugar groups. WaaD, WaaJ and WaaL are some of the enzymes that synthesize the core region and add the O-antigen. WaaL is the ligase that links the O-antigen to the core (Schnittman and Klena, 1993).

LPS partially mediates adhesion to host cells, the first step in bacterial invasion. Indeed, isogenic LPS inhibits adherence of *Shigella* to guinea pig intestinal cells (Izhar et al., 1982). Bacterial adhesion to cells is influenced by surface charge and hydrophobicity (Magnusson et al., 1980). Mutations that result in structural changes to the LPS can also affect the surface charge. Wild-type *Shigella* is uncharged, but strains with rough LPS, which lack the O-antigen, are negatively charged (N. Geldmacher and C. Chaput, unpubl. obs.).
Neutrophils can phagocytose bacteria, form Neutrophil Extracellular Traps (NETs) or degranulate (Nathan, 2006). The effectors of these processes include enzymes and antimicrobial proteins (AMPs) which are stored in granules (Faurschou and Borregaard, 2003). Degranulation is the release of granular proteins into the extracellular space. Some of the granular proteins that we investigate are neutrophil elastase (NE), Bactericidal Permeability Increasing protein (BPI) and the human 18 kDa cationic antimicrobial protein (hCAP18). NE is a protease that specifically degrades virulence factors (Weinrauch et al., 2002). BPI binds to Lipid A (Gazzano-santoro et al., 1992) and is bacteriostatic at low and bactericidal at high concentrations (Mannion et al., 1990; Weiss et al., 1992). hCAP18 is cleaved into the antimicrobial 4.5 kDa C-terminal peptide LL-37 and also binds to LPS (Larrick et al., 1995). Although phagcysis and NET formation kill Shigella, we do not fully understand how degranulation affects this microbe.

Early on in infection, when there are few neutrophils present, the concentration of AMPs released by degranulation is low. We asked how low, sublethal concentrations of AMPs affect Shigella adhesion and invasion of epithelial cells. Here we show that Shigella adhesion and invasion of HeLa cells is greatly enhanced after binding specific antimicrobial proteins released by degranulating neutrophils. This hyperinvasion is caused by cationic proteins, like BPI, that bind to the negatively charged Lipid A. Hyperinvasion is mediated by electrostatic interactions since it requires cationic proteins. Furthermore, a negatively charged LPS mutant shows enhanced hyperinvasion. We propose that during evolution Shigella acquired hyperinvasiveness as a mechanism to escape arriving neutrophils and to increase its virulence in response to cationic AMPs.

Results

Human neutrophil granular proteins enhance Shigella adhesion and invasion

We generated a granule acid extract of purified human neutrophils (hNGP) as described (Weiss et al., 1978) with slight modifications to the protocol. Treating virulent (M90T) and avirulent (BS176) Shigella with 1% hNGP did not affect their viability. Increasing concentrations of the extract resulted in decreasing bacterial viability where 40–50% of the bacteria survived at 10% hNGP (Fig. 1A).

To check how neutrophil granular proteins affect Shigella adhesion of the bacteria to HeLa cells, we incubated Shigella with sublethal concentrations of hNGP before infection. Bacterial adhesion increased from 1% to 14% in a dose-dependent manner (Fig. 1B). Shigella adhesion precedes invasion of HeLa cells, which we tested in a gentamicin protection assay. Incubation of Shigella with hNGP increased invasion up to 30-fold in a dose-dependent manner (Fig. 1C). Similar results were obtained with the human intestinal epithelial cell line HT-29, suggesting that hyperinvasion does not depend on a specific epithelial host cell (Fig. 1D). hNGP induced hyperinvasion independently of the multiplicity of infection (moi) (data not shown).

In our preparation of hNGP, assuming that no proteins are lost during the preparation, there are between 2 and 3 ¥ 10^8 neutrophils per ml. Histological analyses of acute Shigella infections in rabbits (Perdomo et al., 1994a) and humans (Raqib et al., 2000) show that there large numbers of neutrophils infiltrating the inflammatory site. Although there are no quantitative data in shigellosis, in other inflammatory models it is known that up 4 ¥ 10^7 neutrophils per ml are recruited to the inflammatory site (Weinrauch et al., 1995). Since we see an effect of hNGP at 10% or less, the experiments described here are well in physiological range.

Shigella virulence factors delivered by the TTSS are required for hNGP-dependent hyperinvasion, but not for increased adhesion. BS176, a Shigella strain that lacks the virulence plasmid, showed increased adhesion (Fig. 1B) but not invasion (Fig. 1C) after treatment with hNGP. Notably, pre-incubation of Salmonella typhimurium with hNGP did not case hyperinvasion (Fig. 1E). Taken together, these data indicate that hNGP increases Shigella adhesion to the host cell, which in turn results in hyperinvasion.

Degranulation of human blood-derived neutrophils causes hyperinvasion

To check if neutrophil degranulation promotes hyperinvasion, we induced degranulation of increasing numbers of neutrophils seeded in the upper chamber of a transwell system. Neutrophils were stimulated to degranulate with the bacterial peptide fMLF (N-formyl-methionyl-leucyl-phenylalanine). The pore size of the transwell system used allowed the diffusion of degranulated proteins but not the transmigration of neutrophils. One hour after stimulation, we added Shigellae to the lower chamber for 30 min to allow them to mix with the degranulated proteins and then determined invasion in a gentamicin protection assay on HeLa cells already present in the lower chamber (Fig. 2). Bacterial viability was dependent on the number of stimulated neutrophils and decreased by up to 45–55% (Fig. 2B). Notably, upon neutrophil stimulation, Shigella invasion efficiency increased up to 30-fold and this depended on the number of neutrophils. As expected, unstimulated neutrophils did not induce hyperinvasion. These data show that proteins released by neutrophils through degranulation are sufficient to induce hyperinvasion.

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hNGP binding to Shigella causes hyperinvasion

Pre-incubating HeLa cells with up to 10% hNGP did not result in hyperinvasion (Fig. 3A), suggesting that the granular proteins associate with the Shigella surface to induce hyperinvasion. Furthermore, since hNGP enhances adhesion of wild-type and avirulent Shigella, we proposed that the increase of adhesion induced by granular proteins is independent of Shigella virulence factor secretion and is caused by changes in the Shigella surface. If this was the case, removing bound granular proteins should inhibit hyperinvasion. To test this, we first treated Shigella with hNGP and then eluted the bound proteins with 200 mM MgCl₂, competing them out of the bacterial surface (Weiss et al., 1983). Incubation with MgCl₂ did not affect invasion efficiency of untreated Shigella. However, protein elution from the surface of hNGP-treated bacteria reduced hyperinvasion (Fig. 3B), indicating that granular proteins need to be present on the surface of Shigella and not the host cell to cause hyperinvasion.

BPI, hCap18 and LL37 bind to Shigella after hNGP exposure but only BPI induces hyperinvasion

To identify the hNGP protein(s) responsible for hyperinvasion, we incubated Shigella pre-treated with hNGP with high concentrations of MgCl₂ as described above. Eluted proteins were concentrated, resolved by 1D-gel electrophoresis and silver stained (Fig. 4A). The binding of specific proteins (arrows in Fig. 4A) to Shigella depends on the hNGP concentration. The molecular weights of the most abundant proteins suggested that they were BPI and hCAP18. Indeed, Western blot analysis showed that BPI and hCAP18 bind to Shigella (Fig. 4B). Both of these proteins are abundant in neutrophil granules and are released by degranulation (Weiss and Olsson, 1987; Marra et al., 1992). In addition, we confirmed the binding of LL-37, a 37-amino-acid-long cleavage product of hCAP18 (Sorensen et al., 2001), to Shigella.

We next checked whether binding of BPI was sufficient to promote hyperinvasion. Pre-treating Shigella with 1–5 μg ml⁻¹ of recombinant BPI, all sublethal concentrations (Fig. S1A), increased invasion up to 25-fold compared with an untreated control (Fig. 5A). Incubation with LL-37 alone did not increase invasion (Fig. S2A) although killing Shigella (Fig. S2B). Interestingly, BPI and LL-37 acted synergistically since invasion increased 130-fold when Shigella was pre-incubated with 10 μg ml⁻¹ BPI and 5 μg ml⁻¹ LL-37 (Fig. 5A).

To test if other LPS-binding protein induces hyperinvasion, we incubated Shigella with LPS-binding protein (LBP), a serum protein with sequence and structural similarity to BPI (Beamer et al., 1997). Notably, LBP did not cause hyperinvasion (Fig. 5B) and no killing (Fig. S1B) at concentrations between 0.5 and 5 μg ml⁻¹ suggesting that BPI has effects other than just binding LPS.

In contrast to the cationic BPI and LL-37, LBP is neutral. This suggested that positive charges are required in proteins to induce hyperinvasion. Therefore we treated Shigella with poly-lysine (Fig. 5C) or poly-arginine (data not shown), both artificial cationic polypeptides, and determined invasion. Treatment with poly-lysine caused similar killing compared with hNGP (Fig. S1C). Both of these polypeptides induced hyperinvasion at concentration between 1 and 5 μg ml⁻¹, suggesting that the effect of the neutrophil cationic proteins is mediated by charge.

LPS mutants with a negative surface charge show increased hyperinvasion

We reasoned that binding of hNGP to the surface of Shigella might enhance adhesion by facilitating electro-
static interaction between cationic proteins of the hNGP and the negative surface of the host cell. Therefore, we tested whether *Shigella* LPS mutants with different surface charges are hyperinvasive after hNGP treatment. We analysed mutants in the genes *gtrA*, *gtrB*, *rfbA*, *waaL*, and *waaJ*, all of which are defective in rabbit ileal loop colonization (West *et al.*, 2005). Mutants *ΔwaaL* and *ΔwaaJ* have a negative surface charge while wild-type *Shigella* and mutants *ΔgtrA*, *ΔgtrB*, *ΔrfbA* are neutral (N. Geldmacher and C. Chaput, unpubl. obs.). Mutants in *gtrA*, *gtrB*, *rfbA* showed normal invasion and hyperinvasion, while *ΔwaaL* was slightly less invasive than wild-type *Shigella*, but adhesion was not affected. Pre-treatment with hNGP made *ΔwaaL* (Fig. 6A) and *ΔwaaJ* (data not shown) even more hyperinvasive than wild-type *Shigella*. *ΔwaaL* adhered about 5- to 10-fold more efficiently (Fig. 6A) and was two- to threefold more invasive (Fig. 6B) than wild-type *Shigella* M90T when exposed to hNGP. This enhanced invasion is not due to bacterial aggregation, since non-invasive *Shigella* are not entering the host cell by attachment to invasive *Shigella* when mixed infections of hNGP-treated *Shigella* were performed (Fig. S3A). Exposure of
the *waaL* mutant showed increased susceptibility after hNGP treatment (Fig. S2C), indicating that increased binding of AMPs due to the negative charge of the mutant causes more killing. Taken together, these results suggest that *Shigellae* carrying a negative surface charge are more hyperinvasive when treated with hNGP than wild-type *Shigella*, indicating that changes in the surface charge induced by hNGP lead to increased adhesion to and invasion of *Shigella* to host cells.

**Discussion**

Neutrophils are the first cells of the immune system recruited to the site of infection during shigellosis. Neutrophils can attack extracellular *Shigellae* by phagocytosis, NETs and degranulation. Here we provide evidence that

![Graph A](image1)

**Fig. 2.** Neutrophil degranulation causes hyperinvasion. A. Increasing numbers of isolated human neutrophils were stimulated with 1 μM fMLF for 1 h to degranulate in the upper chamber of a transwell system. After degranulation, neutrophils were removed and bacteria were added to the lower chamber. Following incubation with the degranulated proteins intracellular bacteria were enumerated in a gentamicin protection assay. B. Viability of bacteria was determined after exposing *Shigella* to degranulated proteins in the lower chamber and subsequent plating of the bacteria. The unstimulated control contained 1·10^7 neutrophils. This shows that degranulation of neutrophils causes hyperinvasion. Results are shown as a percentage of input bacteria and a representative of three experiments is shown as mean ± SD. *** indicate a p-value < 0.001.

![Graph B](image2)

**Fig. 3.** hNGP binding to bacteria causes hyperinvasion. A. hNGP treatment of host cells does not cause hyperinvasion. HeLa cells were pre-treated with hNGP at the indicated concentrations for 15 min, washed once with DMEM and then infected. Intracellular bacteria were quantified with a gentamicin protection assay. Results are representative of two independent experiments. Data are shown as mean of triplicates ± SD. B. Elution of hNGP proteins from the surface of the bacteria reduces *Shigella* hyperinvasion. *Shigella* were pre-treated with hNGP at the indicated concentrations and then incubated either in PBS or in PBS with 200 mM MgCl2 to elute hNGP proteins. After the treatment, bacterial invasion was measured in a gentamicin protection assay. Results are representative of three independent experiments. Data shown are mean of triplicates ± SD. *** indicate a p-value < 0.001.
Shigella evolved mechanisms to respond to degranulating neutrophils to increase its infectivity.

Perdomo and colleagues elegantly showed that during Shigella pathogenesis, neutrophils are required for the effective infection of the epithelium (Perdomo et al., 1994a). Based on these data, they proposed that neutrophils opened the epithelial barrier by transmigration allowing a massive basolateral bacterial invasion. Our data suggest that besides this function, neutrophils recruited early in infection (Perdomo et al., 1994b) degranulate and provide proteins that allow hyperinvasion. Hyperinvasive Shigella are delivered into the safe haven of the cytosol where they can speedily spread and multiply within the epithelium exacerbating the infection. Our data showing that exposure of Shigella to degranulating neutrophils cause hyperinvasion support the notion that hyperinvasion might occur in vivo. Experiments to determine the individual contribution of these two possible mechanisms are difficult to design because we lack a shigellosis model that recapitulates the spatiotemporal recruitment of neutrophils to the infection site.

Granular proteins promote adhesion independently of the presence of virulence factors. This adhesion requires protein binding to the bacteria, not to the host cell. Our data suggest that the hyperinvasion observed in wild-type Shigella is a direct consequence of the enhanced adhesion. Virulence factors, as expected, are required for invasion. In macrophages, which phagocytose Shigella without the need for an active invasion process, this leads to an increase in cytotoxicity (Fig. S3).

Neutrophil elastase is a granular protein present in hNGP that specifically degrades the virulence factors required for invasion (Weinrauch et al., 2002). Indeed, pharmacological inhibition of NE in hNGP leads to more pronounced hyperinvasion (data not shown). Hence, the inhibitory effect of NE is overridden by hyperinvasion.

We propose that granular proteins mediate hyperadhesion by altering surface charges. BPI, but not LBP, promotes hyperadhesion. Both these proteins bind LPS, their sequence is almost 50% identical and they are structurally similar (Schumann et al., 1990). Interestingly, the isoelectric point of BPI is cationic (9.4) but LBP is slightly anionic (6.3) (calculated using the P.I. tool on the Expasy website) (Gasteiger et al., 2003). Indeed, BPI binding to bacteria depends on electrostatic interactions (Weiss et al., 1983). Interestingly, LL-37, although it is highly cationic (isoelectric point of 10.6) and binds to Shigella, does not promote adhesion on its own, but can enhance BPI activity significantly. This might be because BPI and LL-37 bind to different sites of LPS. These data are consistent with the observation that LL-37 and BPI are also synergistic in killing bacteria (Zarember et al., 2002). Our data showing that the highly cationic poly-Lys and poly-Arg also enhance invasion are in agreement with the notion that hyperinvasion depends on a change in the surface charge.

Interestingly, Shigella is able to suppress expression of antimicrobial peptides in epithelial cells (Sperandio et al., 2008). Among the repressed peptides are HBD3, LL-37 and CCR6. Neutrophils are, in contrast to epithelial cells, transcriptionally nearly inactive and have a short half-life. Therefore, it is very unlikely that Shigella regulates transcription in neutrophils. It is interesting that LL-37, an antimicrobial peptide produced by both epithelial cells and neutrophils, does not facilitate hyperinvasion by itself, but potentiates the effect of BPI. This indicates that antimicrobials produced by different cells might have different roles or even might cooperate in Shigella pathogenesis.

Consistent with the requirement for charge to mediate hyperinvasion, the Δwaal mutant, which lacks the O-antigen, allowing the sterical availability of negative charges in the core region and Lipid A, has increased hyperinvasion. Indeed, wild-type Shigellae are not charged...
(N. Geldmacher and C. Chaput, unpubl. obs.), but its LPS carries several negatively charged groups. These groups are shielded by the O-antigen which includes many polar but uncharged glucosyl-groups, rendering the negative charges less accessible. Indeed, BPI binding depends on the LPS O-antigen chain length in *Escherichia coli* and *Proteus mirabilis* (Capodici et al., 1994). It is important to note that modifications of the LPS also affect the efficiency of the TTSS (West et al., 2005). Additions of groups that render the LPS less negatively charged, such as aminoarabinose, reduce binding of antimicrobial peptides to *Salmonella* (Guo et al., 1998) or *Staphylococcus aureus* (Kraus et al., 2008).

Hyperinvasion is not a general feature of invasive enteric bacteria. In contrast to *Shigella*, hNGP does not cause hyperinvasion of *Salmonella*. This is consistent with the observation that *Salmonella* modifies its LPS to make it less negatively charged which, in turn, leads to less efficient binding of AMPs (Vaara et al., 1979). In contrast to *Shigella* that resides exclusively in the intestinal epithelium, *Salmonella* disseminates rapidly through the bloodstream soon after oral inoculation (Mastroeni et al., 2009). It is interesting to speculate that *Salmonella* probably evolved to express a LPS that does not allow hyperinvasion, since it can disseminate before neutrophils arrive to the site of infection, but it makes it less sensitive to AMPs. It is possible that in *Shigella*, LPS is under conflicting selection pressures. On one hand a negatively charged LPS enhances hyperinvasion. On the other hand, the same characteristic increases the susceptibility to AMPs. In fact, the ΔωaaL mutant is more sensitive to killing by hNGP treatment (West et al., 2005). In conclusion, *Shigella* LPS may have evolved to allow bacterial escape into the safe epithelial niche when neutrophils arrive without rendering *Shigella* too sensitive towards antimicrobial peptides.

**Experimental procedures**

All chemicals were obtained from Sigma unless stated otherwise. The antibody to hCap18 which recognizes both hCap18 and LL37 was a gift of Dr Mona Stahle-Backdahl, Karolinska Hospital, Stockholm, Sweden. The antibody detecting BPI is a kind gift of Jerrold Weiss (Iowa University).
taining 2% FBS during the infection (moi of 100). The Shigellae were centrifuged (300 g) onto the HeLa cells at room temperature for 10 min and then incubated for 30 min at 37°C and 5% CO₂. For adhesion, the cells were washed repeatedly, lysed with 1% Triton/PBS and then the bacteria were plated and counted. For invasion, the cells were washed twice with PBS and incubated in medium containing 100 μg ml⁻¹ gentamicin. One hour later the cells were washed and lysed with 1% Triton/PBS for 5 min and bacteria were plated to determine the intracellular colony-forming units (cfu). For some invasion assays we used a recombinant 25 kDa N-terminal fragment of BPI that has the same antimicrobial and LPS-neutralizing activities as holo-BPI (Meszaros et al., 1993) (kindly provided by Dr Jerrold Weiss, University of Iowa College of Medicine) and purified LL-37 (kindly provided by Dr Mona Stahle-Backdahl Karolinska Hospital, Stockholm, Sweden). Recombinant human LBP was obtained from Alexis Biochemicals.

**Preparation of crude granule extract**

Neutrophils were obtained from buffy coats (German Red Cross) or flushing back leukocyte reduction filters (Meyer et al., 2005) followed by Dextran/Ficoll purification. Blood or elution buffer was mixed with dextran (MP Biochemicals) at 1% final concentration and let stand for 30 min to allow for separation. Afterwards, the leukocyte enriched phase was overlaid on Ficoll-Paque Plus (GE Healthcare) and centrifuged for 30 min at 400 g. Neutrophils and remaining erythrocytes are found in the pellet after centrifugation. Neutrophils were washed once with HBSS (−) and pelleted at 250 g for 10 min. Erythrocytes were lysed by addition of ice-cold pyrogen-free water for 30 s. Then RPMI/10 mM Hepes (pH 7.4) was added to prevent lysis of neutrophils. After centrifugation at 250 g for 10 min, neutrophils were taken up in RPMI/Hepes and counted. To prepare hNGP 5–7 × 10⁶ neutrophils, corresponding to a packed volume of about 0.5 ml in a 15 ml falcon tube, were diluted with 1.1 ml of chilled pyrogen-free water and sonicated for 2 × 30 s using a Bandelin Sono-plus sonicator equipped with a HD2070 sonication tip set to 25% power. Lysis was considered complete when there were no cells sedimenting and the suspension was milky white. Then we added H₂SO₄ to a final concentration of 0.16 M to the homogenized cells and kept on ice, vortexing every 5 min for 30 min. All the following steps were performed in siliconized tubes. The acidic extract was centrifuged at 4°C for 30 min at 16 000 g. The supernatant was dialysed against 2 l of 20 mM sodium acetate buffer (pH 4) for 3 days with two exchanges in 3.5 kDa cut-off dialysis cassettes (Pierce). After dialysis, the extract was centrifuged at 4°C for 5 min at 16 000 g. hNGP is the supernatant of the second centrifugation step. hNGP was kept at 4°C for 1 month. Preparations of human hNGP were standardized by measuring the NE content as described previously (Weinrauch et al., 2002). The anti-S. flexneri sublethal concentrations were determined for each experiment.

**hNGP treatment**

Mid-log-phase Shigella were resuspended in 1 ml of PBS at a density of 1 × 10⁸ containing hNGP or 20 mM sodium acetate buffer, pH 4, as control. hNGP was added last and bacteria were treated for 15 min at 37°C with slow shaking (80 r.p.m.). After treatment, bacteria were centrifuged at 3300 g for 5 min and resuspended in 1 ml of PBS. For elution, bacteria were resus-
pended in 200 mM MgCl₂ and incubated for 15 min at 37°C. After incubation, bacteria were centrifuged at 3300 g for 5 min and resuspended in 1 ml of PBS.

**Transwell system**

The day before the infection we seeded 1 x 10⁵ HeLa cells per well in the lower chamber of a 0.4 µm pore polycarbonate membrane transwell system (Corning). The day of the infection, we purified neutrophils using Histopaque/Percoll as described (Aga et al., 2002), and seeded them in the transwell insert, previously saturated with 10% FBS for at least 1 h, in RPMI containing 10 mM Hepes and without phenol red. We stimulated the neutrophils with 1 µM lipopolysaccharide. After stimulation, the inserts containing the neutrophils were discarded and Shigella grown to mid-logarithmic phase was added at the indicated moi to the lower chamber. After a 30 min incubation, to allow contact between degranulated proteins and Shigella, a gentamicin protection assay was performed as described above. We plated intracellular bacteria 1 h after gentamicin treatment.

**Statistical analysis**

Data were analysed using the Student’s t-test. Data analysis was performed using the Prism 5.0 software (GraphPad). Statistical significance was assumed at a P-value below 0.05.

**Acknowledgements**

This work was supported by the ‘Deutsche Forschungsgemeinschaft’ through the Graduiertenkolleg 1121 ‘Genetic and Immunologic Determinants of Pathogen–Host-Interactions’ and the Max-Planck-Gesellschaft (B.E.). We would like to thank the members of the Zychlinsky lab, Constantin Urban and Franziska Welzel for critical reading of the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Viability of \textit{Shigella} after different pre-treatments. \textit{Shigella} were pre-treated with either (A) BPI, BPI and LL37, (B) LBP or (C) poly-lysine at the indicated concentrations for 15 min at 37°C. After incubation, bacteria were washed and taken up in PBS. The number of viable bacteria was determined by plating dilutions in liquid LB agar.

A. Sublethal killing can be observed after exposure of \textit{Shigella} to increasing concentrations of BPI or BPI and LL-37.

B. LBP does not cause detectable killing of \textit{Shigella} up to 10 μg ml<sup>–1</sup>.

C. Poly-lysine causes comparable killing to hNGP-pre-treatment at the indicated concentrations.

**Fig. S2.** A and B. Exposure of \textit{Shigella} to LL-37 does not cause hyperinvasion. \textit{Shigella} were pre-treated with increasing concentrations of the antimicrobial peptide LL-37. There was no increase in invasion observed at all used concentrations while concentration-dependent killing of \textit{Shigella} could be observed.

C. Viability of M90T and \textit{ΔwaaL} \textit{Shigella} after exposure to hNGP. Wild-type \textit{Shigella} M90T or O-antigen ligase-deficient \textit{Shigella} \textit{ΔwaaL} were incubated with increasing concentrations of hNGP for 15 min at 37°C. \textit{ΔwaaL} mutants are more susceptible to hNGP-induced killing.

**Fig. S3.** A. Hyperinvasion is not caused by aggregation of hNGP-treated \textit{Shigella}. HeLa cells were co-infected with both virulent and avirulent \textit{Shigella} (ratio 1:1) exposed to increasing concentrations of hNGP. A gentamicin protection assay was performed as described to determine the number of intracellular bacteria. Aggregation is not the cause of hyperinvasion, since avirulent \textit{Shigella} are not able to enter the host cell by binding to virulent \textit{Shigella} through aggregation.

B. Macrophages infected with hNGP-treated \textit{Shigella} show enhanced cytotoxicity. Raw264.7 mouse macrophages were infected with hNGP-pre-treated \textit{Shigella} at a moi of 100. With increasing concentrations of hNGP, more macrophages are killed by intracellular \textit{Shigella}.

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