Activation of NF-κB by Neisseria gonorrhoeae is associated with microcolony formation and type IV pilus retraction

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Summary

The early stage of infection with Neisseria gonorrhoeae (Ngo), the causative agent of gonorrhoea, is marked by type IV pilus (Tfp)-mediated attachment and the formation of bacterial microcolonies on epithelial cells. Retraction of the Ngo Tfp generates substantial force on its substrate which can elicit host cell signalling. Here, we observed that this retraction force could also activate nuclear factor (NF)-κB, the central signalling cascade of innate immunity. Using a p65-GFP-expressing epithelial cell line, we show that piliated Ngo induce asynchronous NF-κB activation in infected cells, which is temporally associated with the formation of gonococcal microcolonies. A mutant lacking PilT, an ATPase necessary for Tfp retraction, induced markedly reduced NF-κB activation. This was accompanied by decreased NF-κB target gene transcription and cytokine release. The impaired ability of the pilT mutant to activate NF-κB was compensated by applying mechanical shear stress to the infected host cells, indicating that the mechanical forces generated by retractile pili are involved in the retraction-dependent activation of NF-κB elicited by gonococcal microcolonies. Thus, our work provides evidence for an intriguing relationship between microcolony growth, pilus retraction and host cell signalling, with likely implications with regard to the course of symptomatic versus asymptomatic gonococcal infections.

Introduction

Neisseria gonorrhoeae (Ngo), the causative agent of gonorrhoea and ascending urogenital infections, uses its type IV pili (Tfp) to initiate infection of human mucosal surfaces (Merz and So, 2000). Tfp, hair-like filaments protruding from bacterial cell surfaces, are widespread among Gram-negative bacterial pathogens (Strom and Lory, 1993; Mattick, 2002; Pelicic, 2008). The gonococcal Tfp are unique with regard to their extensive antigenic variability (Meyer et al., 1990) and the production of a relatively conserved tip-located PilC adhesin (Rudel et al., 1995). During the first hours of infection, Ngo attach by forming microcolonies of c. 10–100 bacteria (Merz and So, 2000). Microcolonies are motile structures that fuse to assemble larger forms, which undergo rapid reorganization and bacterial realignment in a seemingly co-ordinated fashion (Higashi et al., 2007). Microcolonies stimulate the recruitment of cortical actin and a variety of signalling proteins in infected cells (Merz and So, 1997), thereby preventing attached bacteria from entering the host cell (Boettcher et al., 2010). Individual bacteria within a microcolony are governed by stochastic processes that elicit the expression of phase-variable, colony opacity-associated (Opa) outer membrane adhesins (Stern et al., 1986). These Opa proteins not only facilitate secondary interactions with the host membrane but also host cell invasion (Merz and So, 2000; Hauck and Meyer, 2003). Tfp exhibit ‘twitching motility’, a phenomenon dependent on the physical extension and retraction of Tfp fibres (Wolfgang et al., 1998; Merz et al., 2000). Tfp retraction is driven by the hexameric ATPase PilT (Merz et al., 2000; Maier et al., 2002; Forest et al., 2004). The ability to retract Tfp fibres constitutes an important feature of Tfp; bacterial mutants deficient in pilT are non-motile, non-competent for DNA uptake, and form disordered microcolonies (Wolfgang et al., 1998; Higashi et al., 2007). In addition, loss of pilT function leads to reduced cortical actin formation and impairs other epithelial responses such as calcium influx and activation of stress kinase pathways (Merz et al., 1999; Ayala et al., 2005; Howie et al., 2005; Lee et al., 2005), indicating pilus retraction is required for optimal signalling to the host cell. Retraction of a single gonococcal pilus...
can generate forces greater than 100 pN (Merz et al., 2000; Maier et al., 2002). Moreover, forces within the nN range can be generated through the formation of Tfp bundles (Biais et al., 2008). The forces exerted on epithelial cells during infection are smaller but persist for at least 24 h (Opitz et al., 2009). Thus, it has been speculated that mechanical forces generated by pilus retraction constitute a new class of bacterial signal for host-cell interactions (Merz and So, 2000). Indeed, pilus retraction force has been shown to activate components of the mitogen-activated protein kinase (MAPK) cascade, leading to cytoprotective effects (Howie et al., 2005).

Infections with Ngo are often associated with a characteristic inflammatory response and a massive infiltration of neutrophils into the infected tissue (Witt et al., 1976; Zenilman, 1993; Ramsey et al., 1995). The initial interaction of Ngo with mucosal epithelial cells triggers the release of inflammatory cytokines and chemokines, which serve to recruit and activate other immune cells to the site of infection (Naumann et al., 1997; McGee et al., 1999; Fichorova et al., 2001; Harvey et al., 2002). The intracellular pathways that finally lead to specific cytokine/chemokine expression patterns involve the activity of the immediate early transcription factor nuclear factor (NF)-κB. NF-κB is a major inflammatory and survival mediator in the cell, controlling transcription of a diversity of genes, including those encoding cytokines and anti-apoptotic machinery (Karin and Lin, 2002). NF-κB exists as a homo- or heterodimer composed of two subunits from a family of five structurally related proteins: p65 (RelA), RelB, c-Rel, p105/p50 (NFKB1) and p100/p52 (NFKB2). The classical heterodimer of p65 and p50, a primary mediator of inflammation, is the most abundant. In non-stimulated cells, the NF-κB complex is localized in the cytoplasm, bound to a family of inhibitory proteins (IκB). A variety of stimuli, including cytokines and bacterial products, lead to the phosphorylation and degradation of the IκB proteins. The NF-κB subunits can then be translocated into the nucleus to trigger transcriptional activation of target genes (Hoffmann and Baltimore, 2006).

Activation of NF-κB during Ngo infection has been demonstrated in different infection models, including epithelial (Naumann et al., 1997; Muenzner et al., 2002) and primary endothelial cells (Muenzner et al., 2001). Here, we provide evidence that the activation of NF-κB by piliated Ngo is strongly associated with the formation and movement of microcolonies. We show that pilus retraction leading to the co-ordinated formation, movement and fusion of microcolonies is necessary for efficient NF-κB activation. Moreover, we show that the mechanical forces exerted on the epithelial cell membrane during Tfp retraction contribute to the activation of NF-κB.

Results

NF-κB activation is associated with gonococcal microcolony formation

To analyse NF-κB activation during infection with Ngo we used A549 SIB01, a human epithelial cell line that stably expresses the NF-κB subunit p65 fused to GFP (Bartfeld et al., 2010). Upon activation with NF-κB stimuli such as TNF-α, translocation of p65-GFP into the nucleus can be observed (Fig. 1A). In addition, Ngo can adhere normally to these cells and form microcolonies (Fig. S1A, central panel). We observed the translocation of p65-GFP into the nucleus upon exposure of A549 SIB01 to different stimuli, including Ngo wild-type (wt) strain MS11, TNF-α, Helicobacter pylori and Legionella pneumophila. However, the translocation profile in response to infection with Ngo differed strikingly in comparison with the other stimuli: despite inducing specific profiles, TNF-α, H. pylori and L. pneumophila all evoked a strong and immediate response, leading to the activation of approximately 60% of cells within the first hour (Fig. 1B; Bartfeld et al., 2009; 2010). In contrast, infection with Ngo resulted in the activation of only a small number of cells within the first hour. Moreover, the percentage of activated cells increased markedly as infection progressed (Fig. 1B). Translocation of p65 in Ngo-infected cells was accompanied by degradation of IκBα, as detected by Western blot (Fig. 1C). Thus, infection of epithelial cells by Ngo results in a unique NF-κB activation profile that differs markedly from the profiles elicited by other molecular and bacterial stimuli. This observation led us to speculate that during the later stages of Ngo infection a process is invoked that might be involved in NF-κB activation.

To investigate this hypothesis, we observed the progression of gonococcal infection using fluorescence live-cell imaging. Time-lapse microscopy over 8 h revealed a transient and asynchronous translocation of p65 in single cells, with all infected cells being activated at least once during the course of infection (Video S1; Fig. 2A). Notably, not only the number of activated cells increased as infection progressed (Fig. 2A) but also the amplitude (i.e. strength) of activation (Fig. 2B). Phase contrast during live-cell microscopy revealed that microcolonies grew through acquisition of single bacteria (Video S1) and possibly through bacterial cell division within microcolonies throughout the course of infection. Furthermore, occasional fusion of small microcolonies to larger structures was observed (Video S1; Fig. 2A), as reported previously (Higashi et al., 2007; Dietrich et al., 2009b). On closer examination of multiple fusion events it became evident that microcolony fusions were rapidly followed by p65 translocation events in cells with contact to the fusing microcolonies, as illustrated for a representative...
microcolony fusion in Fig. 2C. Quantification of microcolony fusion events revealed that the majority of the cells directly beneath the fusing microcolony became activated within 30–60 min after the beginning of a microcolony fusion (Fig. 2D). However, microcolony fusion was not a prerequisite for NF-κB activation, as activation also occurred in cells on which microcolonies were simply growing without fusion (Video S1). Therefore, we hypothesized that microcolony size and/or number of bacteria within a microcolony represented an important criterion for stimulation of NF-κB. Accordingly, the level of NF-κB activation was reduced at lower multiplicity of infection (moi) (Fig. 2E). More importantly, the onset of efficient NF-κB activation in a high percentage of cells was markedly delayed (Fig. 2E), indicating that a minimum number of bacteria within a microcolony was required for this activation. These data show that pilated Ngo activate NF-κB in epithelial cells, but activation increases during later stages of infection, i.e. in correspondence with the steadily growing and fusing bacterial microcolonies.

**NF-κB activation by N. gonorrhoeae is Tfp retraction-dependent**

Following our observed link between NF-κB activation and microcolony formation, and previous work by Higashi et al. (2007) indicating that microcolony movement and fusion require Tfp retraction, we speculated that NF-κB activation could be explained by twitching motility and the forces induced by Tfp retraction. To test this hypothesis, we monitored NF-κB activation during infection with the isogenic retraction-deficient Ngo mutant MS11 ΔpilT (Dietrich et al., 2009a). MS11 ΔpilT formed disordered, aberrant aggregates on A549 cells, distinct from the organized, round-shaped microcolonies of wt MS11 (Fig. S1A). Nevertheless, both MS11 ΔpilT and wt MS11 adhered comparably well to A549 cells. MS11 ΔpilT exhibited a slightly higher adhesion capacity (Fig. S1B), which is consistent with previous reports (Merz and So, 2000) and likely results from the increased autoagglutination observed for pilT mutants (Wolfgang et al., 1998). Invasion indices of both wt and MS11 ΔpilT mutant were low (0.08 ± 0.005% and 0.09 ± 0.08% respectively), but similar for both strains. Regardless of these similarities, NF-κB activation was markedly reduced in cells infected with MS11 ΔpilT as compared with wt MS11 (Fig. 3A). This difference was especially evident at later time points of infection, as the strong NF-κB activation associated with microcolony formation in wt infection was almost completely absent during infection with the pilT mutant (Fig. 3A). Importantly, NF-κB activation by MS11 ΔpilT was moi independent (data not shown), and even though several large pilT mutant aggregates attached to cells late

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**Fig. 1. Neisseria gonorrhoeae activates NF-κB in epithelial cells.**

A. p65-GFP reporter cells A549 SIB01 were seeded on coverslips and either left untreated (non-activated) or activated with TNF-α (10 ng ml⁻¹) for 30 min. Cells were fixed and pictures were taken with a Leica DMR Microscope. Representative activated and non-activated cells are shown. Scale bar = 10 μm.

B. A549 SIB01 cells were infected with N. gonorrhoeae (Ngo) strain MS11, Legionella pneumophila (Lpn) strain Paris, Helicobacter pylori (Hpy) strain P1 (all moi 100), stimulated with TNF-α (10 ng ml⁻¹) or left uninfected (NI). Cells were fixed after the indicated time and p65-GFP translocation was analysed by automated microscopy. Bars represent mean percentages of technical triplicates and the experiment is representative of at least three independent experiments. Standard deviations are not shown for presentation reasons (see Fig. S7 for additional information).

C. Immunoblot analysis of IκBα degradation in A549 cells infected with wt MS11 (moi 100) for 4 h or stimulated with TNF-α for 30 min. Actin serves as a loading control.
Fig. 2. NF-κB activation is associated with the formation of gonococcal microcolonies.
A–C. Infection of A549 SIB01 cells with wt MS11 (moi 50) was monitored over a period of 8 h by live-cell microscopy (see Video S1). Images were captured every 8 min. Phase contrast shows bacteria and formation of microcolonies. Translocation of p65-GFP was detected by immunofluorescence. (A) Excerpts of Video S1 showing transient and asynchronous p65 translocation in A549 SIB01 during late infection. A total of 21 cells were observed by live-cell microscopy. Numbers (No.) of cells with nuclear p65 at the indicated time points are shown. Phase = phase contrast. (B) The average intensity of GFP in the nuclear region of a representative single cell (from Video S1) was measured by Metamorph software. (C) Magnified excerpts of Video S1 showing fusing microcolonies (left panel) and subsequent p65 translocation (right panel) in the cells directly beneath the fusing microcolonies. Microcolonies are indicated by red arrowheads. Phase = phase contrast.
D. Quantification of the temporal correlation between microcolony (MC) fusion and NF-κB activation. Forty-six microcolony fusion events observed in live-cell movies from five independent experiments were analysed and the time between the beginning of the fusion and the maximum of p65 translocation in the cells in direct contact to the fusing microcolony was determined (diff. = difference).
E. Activation of NF-κB is moi-dependent. A549 SIB01 cells were infected with wt MS11 at an moi of 10 or 100. After the indicated time cells were fixed and p65 translocation was analysed by automated microscopy. Error bars = standard deviation of technical triplicates.
infection, p65 was not translocated into the nucleus (Fig. 3B). The differential abilities of MS11 and MS11ΔpilT to stimulate NF-κB activity were also reflected in the pattern of IκBα degradation, as detected by western blot (Fig. 3C). These results were corroborated in a second cell line, ME-180, where similar patterns of p65 translocation and IκBα degradation were observed (Fig. S2A and B). The pilT mutant, MS11ΔpilT, used in this study contains an unmarked 504 bp in-frame deletion, which was shown to have no effect on downstream located genes, such as the pilU gene (Dietrich et al., 2009a). Hence, we can exclude the possibility that the impaired capability of MS11ΔpilT to induce activation of NF-κB is due to a polar effect. Furthermore, it is unlikely that coincidental, secondary mutations are responsible for the observed effects on NF-κB activation (Fig. 3A–C), as a differently generated pilT mutant, MS11pilT:mTnErm (Lee et al., 2005), shows the same aberrant NF-κB activation profile (Fig. S3).

Despite the lack of pilus retraction, MS11ΔpilT induced a weak NF-κB activation early in infection (Fig. 3A). To determine whether this activation was mediated through Tfp-related functions other than retraction (i.e. specific pilus–receptor signalling), NF-κB activation was investigated during infection with the isogenic, non-piliated mutant, MS11ΔpilE. Both pilin expression sites are

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Type IV pilus associated NF-κB activation

Artificial mechanical force restores NF-κB activation by the pilT mutant

To establish whether the mechanical force exerted by retractile pili constitutes the crucial stimulus leading to the activation of NF-κB, we exerted an artificial mechanical force on epithelial cells infected with either wt MS11 or MS11ΔpilT. For this approach we used a cone and plate device to apply shear stress, a mechanical force caused by fluid flow, on the cells (Fig. 4A). It is well known that cells can sense and respond to mechanical stimuli in the form of shear stress, e.g. by changing their morphology, differentiation, function, signalling and gene expression (Davies, 1995; Davies et al., 1997; Ando and Yamamoto, 2009; Stroka and Aranda-Espinoza, 2010). Cone-plate rheological systems have been used and intensively characterized to study these cell responses (Dewey et al., 1981; Bussolari et al., 1982; Davies et al., 1984; Schnittler et al., 1993; Resnick and Gimbrone, 1995; Bongrazio et al., 2000; 2003). The modified cone and plate apparatus used in this study (Fig. 4A) is described in detail in Experimental procedures. Briefly, a rotational flow of culture medium (Newton-type fluid) between a cone and a stationary plate (diameter of 100 mm) is produced by rotation of the cone, and the resulting shear forces acting on the plate can be calculated.

First, the influence of shear stress that was generated using the cone-plate system on the activation of NF-κB in uninfected epithelial cells (A549) was investigated. Here, for technical reasons the degradation of IkBα was used as readout for activation of NF-κB; epithelial cells seeded in 100 mm cell culture dishes were placed under the rotating cone, and hence, could not be transferred to other devices, such as the automated and live cell microscope respectively. Translocation of p65 correlates with IkBα degradation, as shown in Figs 1C, 3C and S2B. In uninfected epithelial cells application of shear stress at rotational speeds covering a range from 70 to 280 rotations per minute (r.p.m.) (corresponding to shear stress levels of approximately 3.15–12.6 dynes cm⁻²) did not induce activation of NF-κB (data not shown). For shear force experiments during bacterial infection the cone was rotated at 140 r.p.m. (6.3 dynes cm⁻²). Higher rotation velocities led to significant detachment of bacteria as determined by adhesion assays (data not shown) and thus were not applied. A549 cells were infected with MS11 or MS11ΔpilT for 3 h, allowing bacteria to form microcolonies and aggregates respectively. Unbound bacteria were removed by medium exchange before exposing cells to continuous fluid shear stress at 140 r.p.m. (for 15 min). Application of shear force restored the ability of the pilT mutant to activate NF-κB, increasing activation to wt levels (Fig. 4B and C). In both non-infected and MS11-infected cells shear force only marginally increased NF-κB activation (Fig. 4B and C). In accordance with previous work demonstrating that pilus retraction force induced activation of MAPKs during infection with Ngo (Howie et al., 2005), we were also able to overcome the impaired capability of MS11ΔpilT to activate diverse MAPK components by application of shear force on pilT-infected cells using the cone and plate system (data not shown).
Shear stress-induced physiological changes in the bacteria could influence their interaction with the host cell, leading to altered cellular reactions such as NF-κB activation. It was considered possible that the shear force applied experimentally by the cone and plate system could enhance bacterial autolysis, resulting in the release of immunostimulating components. Therefore, bacterial autolysis was tested after shear stress application. The level of autolysis was not affected by the application of shear stress (at 140 r.p.m.), as determined by the absence of the exclusively cytosolic bacterial protein Hsp60 (heat shock protein 60) in the external medium as an indicator of autolysis (Fig. S5).

Taken together, these data strongly suggest that the mechanical force of pilus retraction enhances NF-κB activation during gonococcal infection.
Type IV pilus associated NF-κB activation

Pilus retraction-dependent NF-κB activation does not rely on other bacterial surface proteins

Using the cone-plate system we observed that a shear force/laminar flow applied to epithelial cells infected with MS11ΔpilT compensates the deficiency of this retraction-deficient mutant to activate NF-κB, indicating that the mechanical force (exerted on the host surface) generated by pilus retraction strongly contributes to NF-κB activation. However, we cannot exclude that the flow conditions present in the cone and plate device eventually push the bacteria towards the cell surface, heightening the possibility that enhanced NF-κB activation results from an interaction between other bacterial surface proteins and host cell receptors rather than from the simulated Tfp retraction and retraction force. In the case of wt MS11, such artificial positioning should have a minimal influence as the retractile pilus has already established intimate contact between the bacteria and host cell membrane. However, the non-retractile, rigid pili of the pilT mutant could act as a barrier to close physical contact between bacteria and host cell surfaces and any other surface protein interactions between bacteria and host. Thus, we wanted to exclude that the enhanced NF-κB activation observed when shear force was applied during MS11ΔpilT infection could be explained by non-pilus-specific interactions. To investigate this, epithelial cells with adhered wt MS11 and MS11ΔpilT bacteria were centrifuged for 5 min (1000 g) at 4 h p.i. and the translocation of p65-GFP was analysed. Centrifugation is not equal to pilus retraction and thus should only promote the closer contact of MS11ΔpilT to host cells without applying concomitantly similar mechanical stimuli. Using these centrifugation conditions, the centrifugal forces acting on the epithelial cells were comparatively low and did not influence the level of NF-κB activation in uninfected cells, thus excluding the possibility that the centrifugal forces themselves induced activation of NF-κB (Fig. 4D and E). Centrifugation provoked a slight increase in NF-κB activation during infection with both wt MS11 and MS11ΔpilT (Fig. 4D); however, MS11ΔpilT-induced NF-κB activation did not reach the level induced by wt MS11. This is in contrast to our observations of similar NF-κB activation levels by wt MS11 and MS11ΔpilT after application of shear stress (Fig. 4B and C). Moreover, centrifugation at the onset of infection did not alter the ability of wt MS11 and MS11ΔpilT to stimulate NF-κB (Fig. 4E). Thus, we conclude that interaction of other bacterial surface proteins with cellular receptors do not play a significant role in pilus retraction-dependent NF-κB activation.

Pilus retraction regulates expression of NF-κB target genes

NF-κB regulates the transcription of numerous genes involved in diverse cellular processes such as the immune response, proliferation and apoptosis. To determine whether the observed differences in NF-κB activation between wt MS11 and MS11ΔpilT were reflected in the transcription of NF-κB target genes, we used quantitative RT-PCR (qRT-PCR) to analyse the regulation of the NF-κB target genes interleukin (IL)-8, TNF-α, cIAP1, bfl-1, IκBα and A20. Infection of A549 cells with either wt MS11 or MS11ΔpilT induced an upregulation of all tested genes, ranging from twofold (cIAP1) to 600-fold (IL-8) (Fig. 5A). Interestingly, upregulation of all genes, in particular IL-8 and TNF-α, in MS11ΔpilT-infected cells was markedly reduced in comparison with the wt. The impaired capability of MS11ΔpilT to induce upregulation of NF-κB target genes was corroborated in ME-180 cells (Fig. S6). Moreover, MS11 infection of ME-180 cells resulted in a prominent induction of IL-8 secretion, which steadily increased over the infection period, as measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 5B). In comparison, both MS11ΔpilT and MS11ΔpilE induced significantly less IL-8, most notably at later time points of infection (Fig. 5B). These data corroborate our observations of infection-mediated NF-κB activation and highlight the impact of pilus retraction on cellular events such as production of pro-inflammatory cytokines during infection with Ngo.

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Discussion

Here we show that Tfp retraction influences NF-κB activation during infection with Ngo. Infection with the non-retractile pilT mutant leads to a markedly reduced activation of NF-κB, reduced upregulation of NF-κB target genes and impaired release of inflammatory cytokines such as IL-8. Furthermore, we show for the first time that the formation of microcolonies constitutes a critical event for signalling to the host cell, and provide evidence that gonococcal microcolony formation in conjunction with pilus retraction stimulate NF-κB in the infected cell.

Using a highly precise and quantifiable assay system we found that Neisseria induces unexpected and peculiar p65-GFP translocation kinetics as compared with Helicobacter and Legionella (Bartfeld et al., 2009; 2010). While the latter two pathogens induced quick responses with maxima at 1 h post infection, Neisseria caused a weak early activation that increased slowly with the progression of infection. While the early, weak NF-κB activation did not depend on PilT, the much more prominent, late and steadily increasing activation was dependent on pilus retraction. The highest activation level was observed 4 h p.i. and persisted over many hours. In contrast to the long-term activation of NF-κB activation in Legionella infection, which results from a permanent translocation of the p65 subunit into the nucleus (Bartfeld et al., 2009), Ngo induces a transient, but non-permanent, nuclear translocation of p65 in individual infected cells; the long-lasting activation (on the population-level) observed in the present study resulted from the asynchronous and repeated activation of individual cells within the investigated population. It remains an intriguing question why different pathogens induce such remarkably specific profiles of p65 translocation. Since differential dynamics have been shown to induce specific transcriptional responses (Hoffmann et al., 2002; Ashall et al., 2009), we might speculate that each profile could induce adequately adapted host cell responses.

Tfp retraction influences a number of responses in the infected epithelial cell such as calcium flux, stress kinase signalling, cortical actin recruitment and regulation of gene expression (Merz et al., 1999; Ayala et al., 2005; Howie et al., 2005; 2008; Lee et al., 2005). The NF-κB activation observed here may be influenced by pilus retraction in various ways. Since pilus retraction exerts substantial forces on its substrate (Merz et al., 2000; Maier et al., 2002; Biais et al., 2008), a regulatory role of...
this force in the induction of epithelial cell responses has been posited for a number of retraction-dependent host cell functions (Merz et al., 1999; Ayala et al., 2005; Lee et al., 2005). Here, we used a cone-plate device to investigate the role of mechanical forces in NF-κB signalling. Application of shear force (6.3 dynes cm⁻²) redressed the impaired capability of the retraction-deficient pilT mutant to stimulate NF-κB nuclear translocation. Our data strongly support the hypothesis that the mechanical force of pilus retraction induces or enhances cellular signalling cascades that result in the activation of NF-κB. This is consistent with previous studies concerning Ngo PilT (Howie et al., 2005) showing that retraction force simulated by use of a magnetic bead assay (generating tractive forces) accounts for the retraction-enhanced activation of MAPK signalling during infection with Ngo.

Several studies have demonstrated NF-κB activation upon application of mechanical stress, either in endothelial cells (Lan et al., 1994; Nagel et al., 1999; Chen et al., 2003) or in epithelial cells (Li et al., 2003). This corroborates our finding that activation of NF-κB can, in general, be a response to mechanical stimulation. In T84 colorectal epithelial cells, mechanical stimulation via magnetic beads led to activation of MAPK in the absence of bacteria or bacterial components (Howie et al., 2005). In our experiments, NF-κB activation was not influenced in uninfected cells subjected to shear stress; however, the different types of mechanical stress and cell lines, or the duration or frequency of stimuli used in these studies may account for the differences in outcome obtained here, as reported previously for endothelial cells (Jannney and McCulloch, 2007). Nevertheless, mechanical forces in the form of shear stress substituted the missing retractile force in the pilT mutant.

Pilus retraction may also potentially influence NF-κB signalling by bringing the bacterial and host cell membranes into closer contact and thereby promoting interaction of bacterial factors present in the outer membrane with their appropriate cellular receptor. However, centrifugation of bacteria onto the cell, which promotes the proximity of the bacteria and host cell irrespective of pilus retraction, only slightly increased NF-κB activation, and did not abolish the striking differences in NF-κB induction between wt MS11 and pilT mutant. Thus, secondary receptor engagement most likely does not constitute the key reason for the high impact of pilus retraction on NF-κB signalling.

In addition to the efficient NF-κB activation induced by pilus retraction, we also observed a weak, retraction-independent activation during early infection time points. This activation was also induced by a non-piliated pilE mutant as well as non-viable bacteria, indicating that it was independent of specific pilus–receptor interaction and possibly caused by an outer membrane factor. Likely candidates include the gonococcal outer membrane proteins PorB porin and Lip lipoprotein, known to stimulate NF-κB activation in epithelial cells (Fisette et al., 2003; Binnicker et al., 2004). Other likely contenders include peptidoglycan: the outer membrane vesicles from Gram-negative bacteria such as Ngo might deliver peptidoglycan to NOD1 in epithelial cells, thereby leading to the upregulation of NF-κB-dependent responses (Kaparakis et al., 2010). Previous experiments showing that bacterial lipopolysaccharide does not induce nuclear translocation of p65-GFP in the A549 SIB01 cells used in this study (Bartfeld et al., 2010), renders Neisseria lipooligosaccharide as an unlikely inducer of NF-κB activation. Interestingly, using the reporter cell line, A549 SIB01, we could show that non-piliated Opa-expressing bacteria induced an early, very strong NF-κB activation, completely distinct from the activation profile induced by piliated Ngo, indicating that Opa-mediated activation occurred via immediate interaction and signalling between the Opa proteins and their receptor. Opa-dependent activation of NF-κB has been previously shown for other cell lines (Naumann et al., 1997). Furthermore, since the piliated strains MS11 and MS11ΔpilT did not express Opa proteins during infection, we can rule out the possibility that Opa proteins induce the early, weak and retraction-independent NF-κB activation.

The function of microcolony formation by Neisseria has been an enduring open question. Previous work has shown that microcolony formation enables Neisseria to resist the mechanical force of body fluids (Mikaty et al., 2009). However, it may also be possible that Neisseria receive additional benefits by forming microcolonies, such as altered host cell responses. Here, the presented data suggest that not only the number of bacteria attaching to a cell is critical for NF-κB activation, but also their co-ordinated interaction within growing microcolonies is particularly important. Our live-cell experiments reveal that particularly after the fusion of two microcolonies, which likely generates higher forces on the cell membrane temporarily, p65 was immediately translocated to the nucleus. Further, higher moi did not lead to immediate activation but instead to a slowly increasing level of activation. In addition, the pilT mutant, while deficient in pilus retraction, was still able to form large aggregates on host cells; however, these bacterial aggregates did not activate NF-κB efficiently. This leads us to conclude that a correlation between microcolony growth, pilus retraction and NF-κB activation exists. We hypothesize that only the concerted pilus retraction of many bacteria within a microcolony exerts sufficient local tensile forces on the host cell to efficiently activate or enhance NF-κB signalling. Whether a defined threshold level of retraction force needs to be exceeded before signalling occurs, and if so what this level is, remains to be determined. Unfortu-
nately, the cone-plate device does not support accurate force calculations, as only the average (macroscopic) shear force rate on the plate surface can be calculated and not the exact force acting on one given cell (Dewey et al., 1981). In support of a threshold effect, however, we found that application of 50% lower shear stress levels (i.e. through reduction from 140 r.p.m. to 70 r.p.m.) during MS11/spiT infection was not capable of activating NF-κB (data not shown).

Taken together, our study shows that the formation of microcolonies by Tfp-expressing Ngo constitutes a crucial event early in infection. It promotes the co-ordinated interaction of bacteria and thus may lead to synchronized bacterial activities; for instance, the concerted retraction of pili. In this way bacteria can elicit signalling cascades leading to the activation of NF-κB. Through regulation of this global transcription factor gonococcal microcolonies may regulate diverse host cell functions, including apoptosis and induction of the innate immune response (e.g. through regulation of cytokine expression), and thus influence how the infection process proceeds. Since infections with Ngo are usually associated with a strong local inflammation but can also remain asymptomatic, determination of the bacterial features that influence host cell responses, such as cytokine production, will be of particular relevance for future studies.

Experimental procedures

**Cell lines, bacterial strains and infections**

The human alveolar carcinoma cell line A549 (ATCC CCL-185) and its derivative A549 SIB01 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamate and 1 mM Na-Pyruvate (Invitrogen). The human cervix carcinoma cell line ME-180 (ATCC HTB-33) was grown in McCoy's 5A medium supplemented with 10% FCS. Two hours before infection, medium was changed to serum-free medium. Strains MS11/pilT (N1147) (Dietrich et al., 2009a), MS11/pilE (MS11-307) (Merz et al., 1996) and MS11/pilT::TnEm (Lee et al., 2005) are derivatives of Ngo strain MS11-A, which expresses Tfp but not Opa (Segal et al., 1986). The non-piliated Opa<sub>Δ</sub>-expressing GC strain (N313) has also been described (Kupsch et al., 1993). Piliation and Opa phenotypes were monitored by light microscopy of colony morphology. Bacteria were passaged twice on agar plates, after 24 h and 16 h, respectively, and only non-Opa-expressing (wt MS11, MS11/pilT and MS11/pilE) and pilated (wt MS11 and MS11/pilT) bacteria were used for infection experiments. To test bacteria for Opa expression during infection, supernatants were removed at the indicated time points, and infected cultures were washed and lysed for 15 min with 0.5% saponin (Serva). Serial dilutions of the lysates were plated on GCB agar to check colony morphology after 24 h growth. For infection experiments, bacteria were harvested in serum-free cell culture medium and added to sub-confluent cells at an moi of 100, unless otherwise stated. When indicated, infection was promoted by centrifugation at 1000 g for 5 min at 37°C. Stimulation of the cells with TNF-α (10 ng ml<sup>−1</sup>) was performed for 30 min, or as indicated. For heat inactivation, bacteria were resuspended and subjected to 56°C for 30 min. The effectiveness of heat killing was confirmed by plating bacteria on agar plates. Adherence of bacteria to cells and microcolony formation was observed by light microscopy after washing three times with phosphate-buffered saline (PBS).

**Automated microscopy and image analysis**

Generation of the stably p65-GFP-expressing cell line A549 SIB01 and automated image analysis to quantify nuclear translocation of p65-GFP is described elsewhere (Bartfeld et al., 2010). Briefly, A549 SIB01 cells, seeded in 24-well plates, were infected with Ngo strains at an moi of 100. After the respective incubation time, cells were fixed with 100% ice-cold methanol, stained with Hoechst 33342 (2 μg ml<sup>−1</sup>) and stored in PBS. Images of ~1000 cells per well were acquired using the automated microscopy system Scan∧R (Olympus) and translocation of p65-GFP to the nucleus was quantified using the Scan∧R image analysis software (Olympus). The software defines nuclear and cytoplasmic regions of each cell using Hoechst staining and measures intensities of p65-GFP in these regions. Cells with nuclear p65-GFP above the defined threshold were termed ‘cells with nuclear p65-GFP’ and the percentage of cells with nuclear p65-GFP per well was calculated.

**Live-cell microscopy**

A549 SIB01 cells were grown in 3.5 cm<sup>2</sup> glass bottom dishes (MatTek) overnight under standard conditions. Two hours before infection, medium was changed to serum-free medium without phenolred (Gibco, Invitrogen). For infection, bacteria were harvested in the same medium and added to the cells at an moi of 50. Infected cells were placed in a humidified incubation chamber at 37°C and 5% CO<sub>2</sub>. Images were obtained every 8 min using the VT-Infinity system (VisiTech systems), with the first image taken ~10 min after the onset of infection. The system consists of an Olympus IX81 (Olympus), a VT-Infinity galvo scanner confocal head (VisiTech systems) and a Hamamatsu C9100-02 CCD camera (Hamamatsu Photonics K.K.). Images were acquired and processed using Metamorph (Universal Imaging Corporation) software.

**Immunofluorescence microscopy**

A549 SIB01 cells were seeded in 96-well plates and either left untreated or incubated with TNF-α (10 ng ml<sup>−1</sup>) for 30 min. Cells were fixed with 4% paraformaldehyde (Pfa) for 20 min, washed and stored in PBS. Images were taken with a Leica DMR microscope. For investigation of p65 translocation in ME-180 cells, cells were grown on 12 mm coverslips and infected with MS11 or MS11/pilT for 4 h, or incubated with TNF-α (10 ng ml<sup>−1</sup>) for 30 min. The coverslips were washed in PBS, fixed with 4% Pfa for 20 min at room temperature, and washed again. Permeabilization of cells and blocking was performed with 0.1% Triton X-100 and 0.2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. The primary antibodies mouse anti-p65
(Santa Cruz) and rabbit anti-N. gonorrhoeae (GC) (US Biological) were diluted (1:100) in blocking solution, added to samples and incubated for 1 h at room temperature. Coverslips were washed and incubated with 1:100 diluted Cy2-labelled goat anti-mouse and Cy3-labelled goat anti-rabbit secondary antibodies (Jackson Immuno Research) for 1 h at room temperature. For nuclear staining, Draq5 (Biostatus) was added to the secondary antibody solution (1:1000). The samples were rinsed with PBS, mounted on glass slides with mowiol and analysed by fluorescence microscopy using a Leica TCS SP-1 microscope, equipped with an argon/krypton mixed gas laser source (Leica). Negative control samples were processed as described above in the absence of primary antibodies.

Shear stress experiments

For application of artificial mechanical forces on cells a cone and plate system was used. The working principle and basic construction of a cone and plate device is similar to a cone-plate viscometer and is described in detail elsewhere (Dewey et al., 1981; Schnittler et al., 1993). The modified cone and plate apparatus used in this study consisted of a stationary plate and a movable cone (base diameter of 96 mm) with a cone angle of 1°. A 100 mm tissue culture dish can be placed onto the stationary plate and fixed with a steel ring. The cone and the stationary plate are fabricated of stainless steel. Fine adjustment of the distance between plate and tissue culture dish, respectively, and the cone is carried out with a micrometric screw and a measuring spindle connected to a stationary digital sensor. Rotation of the cone is driven by a micromotor (Faulhaber) with a 10 W power supply. Rotational speeds between 20 r.p.m. and 375 r.p.m. are adjustable. The whole system was placed in an incubator and experiments were carried out at 37°C.

The wall shear stress (τw) was calculated according to the formula

\[ \tau_w = (\omega/\alpha) \eta \]

where \( \omega \) (rad s\(^{-1}\)) is the angular velocity, \( \alpha \) (rad) the cone angle and \( \eta \) (dyn s cm\(^{-2}\)) the fluid viscosity. With a medium viscosity of 0.0075 dyn s cm\(^{-2}\) (at 37°C), the given cone angle of 1° and an angular velocity of \( \leq 14.7 \) (rad s\(^{-1}\)) (rotational speed: \( \leq 140 \) r.p.m.), the Re (Reynolds number) is \( < 1 \), indicating laminar flow conditions over the whole tissue culture dish. By using higher angular velocities and rotational speeds (> 140 r.p.m.), respectively, this calculation gives an average value of the applied shear stress, since the occurrence of local turbulent flows could lead to locally increased forces (Dewey et al., 1981; Sdougos et al., 1984).

For infection experiments with the cone and plate system, A549 cells, seeded into 100 mm Primaria tissue culture dishes (Becton Dickinson Labware), were infected with wt MS11 or MS11ΔpelT (moi 100). After the indicated infection time unbound bacteria were removed and 10 ml of fresh serum-free cell culture medium was added. The open cell culture dishes were positioned and fixed in the cone and plate device, and the cone was carefully moved down till a distance between the apex and the bottom of the culture dish of 2 mm. Cells were then exposed to a continuous fluid shear stress for 15 min at 140 r.p.m. After repeated medium exchange to remove bacteria detached by the shear stress, the cells were incubated for additional 20 min at 37°C. Cells were then collected and degradation of IĸBα or phospho-p38 and JNK, respectively, was analysed by immunoblot and compared with cells not exposed to shear stress but otherwise treated identically.

RNA isolation and real-time qRT-PCR

Cells were infected with bacterial strains for 3 h at an moi of 100. Total RNA was isolated from infected cultures using the RNeasy kit (Qiagen), and potential traces of DNA were removed by an additional on-column digestion with DNase I, according to the manufacturer’s instruction (Qiagen). RNA concentration was measured using NanoDrop 1000 (Thermo Fisher Scientific) and RNA quality was accessed using a 2100 Bioanalyzer (Agilent Technologies). One microgram of total RNA was reverse transcribed to generate cDNA, using the iScript cDNA synthesis kit (Bio-Rad). As a control, parallel samples were run without reverse transcriptase. Quantitative RT-PCR using QuantFast Sybr Green PCR Kit (Qiagen) was carried out on the ABI Prism 7900HT sequence detection system (Applied Biosystems). Housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for relative quantification. RT reactions were performed in triplicates for each oligonucleotide pair. Data were analysed using SDS 2.2.2 (Applied Biosystems) software, and relative quantification of gene transcription was performed using the comparative Ct (threshold cycle) method.

IL-8 quantification

ME-180 cells were infected with bacterial strains at an moi of 100. After the respective incubation time, the supernatant was removed for IL-8 measurement. IL-8 was quantified by sandwich ELISA using HU IL-8 Cytoset Kit (Biosource), according to the manufacturer’s protocol. Recombinant IL-8 was used as a standard. Detection was performed with 3,3′,5,5′-tetramethylbenzidine peroxidase substrate solution (KPL). The plate was measured at the ELISA reader Spectra Max 190 (Molecular Devices) at 450 nm.

Immunoblotting

Cells were lysed with 1× SDS loading buffer and boiled for 10 min at 95°C prior to analysis by SDS-PAGE on a 12% polyacrylamide gel as described previously (Laemmli, 1970). The separated proteins were transferred to PolyScreen PVDF transfer membranes (Perkin Elmer), which were blocked by incubation for 1 h at room temperature in TBST buffer with 3% BSA. Membranes were probed with the following primary antibodies according to manufacturer’s protocol: mouse IĸBα (Cell Signaling), mouse/rabbit phospho-p38 MAPK (Thr108/Tyr182) (Cell Signaling), mouse/ rabbit phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling), mouse Hsp60 (Alexis Biochemicals), rabbit anti-GC (US Biological) and mouse β-actin (Sigma) as loading control. Antibody detection was carried out using horseradish peroxidase (HRP)- conjugated secondary antibodies (Amersham Biosciences), and a Western Lightning Plus-ECL, Enhanced Chemiluminescence substrate (Perkin Elmer). Blots were quantified using ImageJ software (v1.44a).

Adhesion and invasion (gentamicin protection) assay

A549 cells were grown to 80% confluency in 24-well plates 24 h prior to infection. Adhesion and invasion was measured as

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described previously (Shaw and Falkow, 1988; Waldbeser et al., 1994). Briefly, bacteria were added to epithelial cells at an moi of 50 and incubated for 4 h. After removal of supernatant the infected cultures were washed with medium. Supernatants and washes of infected cells were collected and saved for plating. For measurement of adhesion, washed cells were directly lysed in preheated 1% saponin/DMEM solution. To determine invasion rates, infected cells were incubated for additional 2 h with 50 µg ml⁻¹ gentamicin/DMEM prior to lysis by 1% saponin solution. Serial dilutions of the supernatants/washes and lysates were plated on GCB agar, and DMEM prior to lysis by 1% saponin solution. Serial dilutions of the supernatants/washes of infected cultures were incubated for additional 2 h with 50 µg ml⁻¹ saponin/DMEM solution. To determine invasion rates, infected cultures were washed with medium. Supernatants and washes of infected cells were collected and saved for plating.

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Supporting information

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

Video S1. Infections with Ngo induce microcolony associated asynchronous nuclear translocation of p65-GFP. p65-GFP-expressing A549 SIB01 cells were infected with Ngo wt strain MS11 (moi 50) and monitored with live-cell microscopy. Lower left panel shows overlay of p65-GFP (green) and bright field. Upper left panel shows bright field only, and upper right panel shows p65-GFP only. Red arrows indicate moving and fusing microcolonies. Images were acquired every 8 min and the elapsed time is shown in h:mm (bottom right).

Fig. S1. Adhesion of Ngo wt MS11 and MS11.pilT to A549 alveolar epithelial cells. A. A549 cells were infected with wt MS11 and MS11.pilT (moi 100) and formation of microcolonies was monitored by light microscopy after 2 h of infection. Representative microcolonies are indicated by black arrowheads.

B. Adhesion indices of wt MS11 and MS11.pilT on A549 cells. A549 cells were infected for 4 h with wt MS11 and MS11.pilT (moi 50) and the number of cell-associated bacteria was determined by adhesion assays. Results represent mean ± standard deviation of two independent experiments performed in technical duplicate.

Fig. S2. Ngo activates NF-κB in ME-180 cells in a Tfp retraction-dependent manner.

A. ME-180 cells were infected for 4 h with wt MS11 or MS11.pilT (moi 50) or stimulated with TNF-α for 30 min. Cells were fixed and stained with p65 antibody (green), gonococcal (GC) antibody (red) and Draq5 (blue), and analysed by confocal fluorescence microscopy. The number of activated cells (defined as cells with a nuclear : cytoplasm green signal ratio ≥ 1) was quantified by manual counting of ~50 cells per condition. Images are representative of three experiments.

B. Immunoblot analysis of IκBα degradation in ME-180 cells infected with wt MS11 or MS11.pilT (moi 100) for 240 min or stimulated with TNF-α for 30 min. Actin serves as a loading control. Densitometric data of IκBα levels normalized to the corresponding actin levels are given below. The value for the non-infected (NI) control was set to 1.

Fig. S3. Comparison of NF-κB activation levels induced by two different Ngo pilT mutants. A549 SIB01 cells were infected with wt MS11, MS11.pilT or MS11.pilT:mTnErm (Lee et al., 2005) at an moi of 100. Cells were fixed at the indicated times and p65-GFP translocation was analysed by automated microscopy. Data are mean ± SEM of three independent experiments.

Fig. S4. Heat inactivation abolishes bacterial adherence to cells. Bacteria (wt MS11 and MS11.pilT) were heat-treated for 30 min at 56°C or left untreated and then added to A549 cells at an moi of 100. After 2 h unbound bacteria were washed away and adherence of bacteria to cells was monitored by light microscopy.

Fig. S5. Exposure of shear stress to Ngo does not stimulate autolysis. After infection of A549 cells with wt MS11 or MS11.pilT (moi 100) and application of shear stress (6.3 dynes cm⁻²; 15 min) (as described in Experimental procedures) the supernatant culture medium was collected and filtered to remove intact bacteria. Autolysis was measured by immunoblot detection of the cytosolic bacterial protein Hsp60 in the medium. Bacteria directly lysed in loading buffer serve as a positive control.

Fig. S6. Regulation of NF-κB target genes in ME-180 cells is influenced by Tfp retraction. ME-180 cells were infected with wt MS11 or MS11.pilT (moi 100) for 3 h. Cells were harvested, RNA isolated and upregulation of indicated genes was quantified by real-time qRT-PCR. mRNA levels are relative to GAPDH mRNA. Non-infected mRNA level was set to 1. Values and ± SEM are calculated from the results of two independent experiments.

Fig. S7. NF-κB activation in MS11.pilT-infected A549 SIB01 cells induced by different stimuli. Data presented in Fig. 1B with indicated standard deviations. Bars represent mean percentages ± standard deviations of technical triplicates and the experiment is representative of at least three independent experiments.

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