Stimulation of the cytosolic receptor for peptidoglycan, Nod1, by infection with Chlamydia trachomatis or Chlamydia muridarum

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Summary

Infection of epithelial cells by the intracellular pathogen, Chlamydia trachomatis, leads to activation of NF-xB and secretion of pro-inflammatory cytokines. We find that overexpression of a dominant-negative Nod1 or depletion of Nod1 by RNA interference inhibits partially the activation of NF-xB during chlamydial infection in vitro, suggesting that Nod1 can detect the presence of Chlamydia. In parallel, there is a larger increase in the expression of pro-inflammatory genes following Chlamydia infection when primary fibroblasts are isolated from wild-type mice than from Nod1-deficient mice. The Chlamydia genome encodes all the putative enzymes required for proteoglycan synthesis, but proteoglycan from Chlamydia has never been detected biochemically. Since Nod1 is a ubiquitous cytosolic receptor for peptidoglycan from Gram-negative bacteria, our results suggest that C. trachomatis and C. muridarum do in fact produce at least the rudimentary proteoglycan motif recognized by Nod1. Nonetheless, Nod1 deficiency has no effect on the efficiency of infection, the intensity of cytokine secretion, or pathology in vaginally infected mice, compared with wild-type controls. Similarly, Rip2, a downstream mediator of Nod1, Toll-like receptor (TLR)-2, and TLR4, increases only slightly the intensity of chlamydial infection in vivo and has a very mild effect on the immune response and pathology.

Thus, Chlamydia may not produce sufficient peptidoglycan to stimulate Nod1-dependent pathways efficiently in infected animals, or other receptors of the innate immune system may compensate for the absence of Nod1 during Chlamydia infection in vivo.

Introduction

Chlamydia is a highly successful pathogen that causes a wide variety of diseases in humans and other species. Different strains of C. trachomatis are responsible for infections of ocular tissue, the leading cause of preventable blindness, and other strains represent the most common sexually transmitted bacterial pathogens in humans (Thylefors et al., 1995; Gerbase et al., 1998; Belland et al., 2004). These Gram-negative, obligate intracellular bacteria infect mainly epithelial mucosa and exist in two developmental forms: the infectious extracellular elementary bodies (EB) attach to the host cell and invade the cell through entry vacuoles that avoid fusion with lysosomes (Moulder, 1991; Hackstadt, 1999; Wyrick, 2000). Within 2–6 h, the EB differentiate morphologically into multiplyative intracellular reticulate bodies (RB), although a subset of chlamydial immediate-early genes are already expressed by 1 h post infection (Belland et al., 2003; Nicholson et al., 2003). The RB proliferate within the same membrane-bound vacuole, called an inclusion, and begin redifferentiating into EB within a day. Finally, after 2–3 days, the EB are released from the infected cell in order to initiate a new cycle of infection.

An inflammatory response is necessary to eliminate primary C. trachomatis infection, but chronic inflammation leads to the long-term damage observed in trachoma and chlamydial sexually transmitted disease (Rank, 1999; Ward, 1999). The pathogenesis of chlamydial infection remains poorly characterized in humans, and most of our knowledge of acute infection has been obtained from the mouse model of vaginal infection (Barron et al., 1981) with the mouse pneumonitis (MoPn) serovar of C. trachomatis [also known as C. muridarum (Everett et al., 1999; Schachter et al., 2001)]. In mice, C. muridarum infection is ultimately resolved through an adaptive CD4+ TH1 response (Rank et al., 1992; Cain and Rank, 1995; Perry et al., 1997). The adaptive response is in turn initiated by
the innate immune system, which represents the first line of defence against pathogens and can be activated rapidly following infection (Janeway and Medzhitov, 2002). The main cells of innate immunity are phagocytic cells such as neutrophils, macrophages and dendritic cells. These cells can discriminate between non-infectious self and infectious non-self by utilizing a limited number of germline-encoded pattern recognition receptors (PRR), which are comprised of families of soluble extracellular, cell-surface bound, and cytosolic receptors. The PRR have been shown to interact with pathogen-associated molecular patterns (PAMP), which are conserved components produced by microbial pathogens but not the host (Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002), although under some circumstances they may also be activated by host cell-derived ligands such as mRNA and DNA (Boule et al., 2004; Karikó et al., 2004).

In contrast to the well-defined roles of Toll-like receptors (TLRs) in cells of the myeloid lineage, the roles played by TLRs in epithelial cells, the preferential target cells of Chlamydia, remain poorly understood (Cario et al., 2000; Girardin et al., 2002; Bäckhed and Hornef, 2003). Although most non-sterile epithelial surfaces such as the gastrointestinal and lower female genital tract are constantly exposed to extracellular bacteria and their products, they remain largely refractory to the presence of these potentially inflammatory mediators, presumably as a way to prevent the induction of a perpetual immune response against the resident bacterial flora. However, epithelial cells are able to initiate an inflammatory response after invasion by the intracellular bacterial pathogens Shigella flexneri and C. trachomatis (Rasmussen et al., 1997; Philpott et al., 2000), demonstrating that epithelial cells are capable of detecting the presence of S. flexneri and C. trachomatis PAMP. Infection of cervical epithelial cells with C. trachomatis leads to the secretion of pro-inflammatory cytokines including interleukin-1 (IL-1), IL-8, IL-6, GM-CSF and GRO-α. Unlike the rapid cytokine response following infection with other invasive bacteria such as S. flexneri, the cytokine response to C. trachomatis infection does not occur until a day after infection (Rasmussen et al., 1997), suggesting that chlamydial PAMP are either weakly immunostimulatory or may not be present in sufficiently high concentrations at the beginning of the chlamydial developmental cycle to activate surface-bound or cytosolic PRR. However, the mechanism by which the presence of the intracellular Chlamydia may be sensed by the epithelial host cell has not yet been determined.

A likely mechanism for intracellular recognition of Chlamydia has been recently described. Nucleotide binding oligomerization domain (NOD) proteins, including Nod1 and Nod2, are members of an emerging family of cytosolic PRR that have the ability to detect bacterial components (Chamaillard et al., 2003a; Inohara et al., 2005). Nod1 is expressed ubiquitously, whereas Nod2 is restricted mainly to monocytes. Nod1 is composed of an N-terminal caspase-recruitment domain (CARD), a centrally located NOD, and multiple C-terminal leucine-rich repeats (LRR). In epithelial cells, Nod1 activates the transcription factor NF-κB, which is usually associated with an inflammatory response, and c-Jun N-terminal kinase (JNK), which is involved in the stress response of cells to different stimuli (Girardin et al., 2001). Several reports have suggested that following PAMP recognition, oligomerization of either Nod1 or Nod2 induces the recruitment of the RIP-like interacting CLARP (caspase-like apoptosis-regulatory protein) kinase (Rip2, also known as RICK or CARDIAK) through homophilic CARD–CARD interactions (Bertin et al., 1999; Inohara et al., 1999). The interaction between Nod1 and Rip2 induces the recruitment of the IκB kinase complex (IKK), which phosphorylates IκB, thereby promoting the activation of NF-κB.

Recently it was shown that Nod2 is a general sensor of PGN through recognition of muramyl dipeptide (Girardin et al., 2003a; Inohara et al., 2003), which is common to both Gram-positive and Gram-negative bacteria. Nod1, on the other hand, is a more selective PRR, as it detects diaminomypetile (DAP)-containing PGN (specifically, the dipeptide motif γ-D-glutamyl-mesoDAP), which is found mostly in PGN of Gram-negative bacteria (Chamaillard et al., 2003b; Girardin et al., 2003b). PGN has never been detected biochemically in chlamydiae, although it is possible that the bacteria may produce small but functional amounts of PGN (Chopra et al., 1998; Ghuyse and Goffin, 1999). It is also not known whether chlamydial products from C. trachomatis or C. muridarum or the whole bacteria can interact with Nod1. We have therefore addressed the question of whether chlamydiae can activate cell-signalling pathways leading to pro-inflammatory cytokine secretion after stimulation of Nod1. We show here that Nod1 is involved in NF-κB activation by C. trachomatis and C. muridarum in epithelial cell lines, implying that chlamydiae do in fact produce evolutionarily conserved PGN.

**Results**

*Activation of the eukaryotic transcription factor NF-κB by C. trachomatis and C. muridarum*

Chlamydial infection induces host epithelial cells to upregulate the expression of several pro-inflammatory cytokine genes – including IL-1, IL-8, IL-6, GRO-α, GM-CSF – in a time- and dose-dependent manner (Rasmussen et al., 1997). As NF-κB is an important transcriptional regulator of genes encoding cytokines and chemokines and Nod1 stimulation leads to NF-κB activation, we first measured the time-course of NF-κB activation during infection of a
human endocervical epithelial cell line with sexually transmitted strains of *Chlamydia*. The NF-κB response was investigated by transfecting HeLa cells transiently with an NF-κB-luciferase reporter plasmid (Ten et al., 1992; Munoz et al., 1994) and subsequently measuring luciferase activity as a function of time after infection with either the lymphogranuloma venereum serovar L2 (LGV2) of *C. trachomatis* or *C. muridarum*. There was a low basal activity of NF-κB in uninfected cells, which became significant within 4 h of infection and was maximal at 6–8 h (not shown). The extent of NF-κB activation invariably decreased after 8 h post infection, and returned to basal levels within 24 h of infection. Similar kinetics of NF-κB activation were observed during infection with either *C. muridarum* or *C. trachomatis* LGV2, implying that differences in the length of the developmental cycle of these chlamydiae should not have measurable effects on the kinetics of cytokine secretion by infected epithelial cells. Subsequent experiments on the role of potential PRR in mediating NF-κB activation were therefore performed at 6 h post infection.

**Influence of the cytosolic PRR, Nod1, on NF-κB activation during Chlamydia infection in epithelial cells**

Our aim was then to identify the signalling pathway leading to NF-κB activation in HeLa cells during infection by *Chlamydia*. HeLa cells express mRNA for all 10 human TLRs, including TLR2 and TLR4 (Nishimura and Naito, 2005). However, they are largely refractory to stimulation by extracellular bacteria and their products, although they respond to invasive strains of *S. flexneri* with activation of NF-κB and release of IL-8 (Philpott et al., 2000). An intracellular TLR-like protein, Nod1, has been identified in epithelial cells and shown to promote these responses during infection with *S. flexneri* (Girardin et al., 2001). Messenger RNA for Nod1 is also present in murine oviductal epithelial cell lines (Derbigny et al., 2005). We therefore investigated whether Nod1 could be involved in NF-κB activation following infection of HeLa cells by the intracellular pathogen *Chlamydia*.

HeLa cells were transiently transfected with a vector construct expressing a Nod1 molecule lacking its CARD domain (ΔCARD Nod1). As the CARD domain is necessary for NF-κB activation, the ΔCARD Nod1 construct acts as a dominant-negative inhibitor of NF-κB induction by *Chlamydia*. In fact, overexpression of ΔCARD Nod1 led to dose-dependent inhibition of NF-κB activation in cells infected with *C. muridarum* (Fig. 1) or *C. trachomatis* LGV2 (not shown) for 6 h. Although the effect was partial, it was reproducible and statistically significant. Thus, transfection with 100 ng of ΔCARD Nod1 led to a 26% decrease in NF-κB activity in infected cells, compared with vector alone. At these Nod1 dominant-negative concentrations, there was essentially no effect of transfection on NF-κB activation due to tumour necrosis factor (TNFα) treatment (not shown), in agreement with previous studies (Girardin et al., 2001).

Given the mild effect observed due to overexpression of the Nod1 dominant-negative construct, we confirmed whether Nod1 plays a role in NF-κB activation by depleting Nod1 in HeLa cells by RNA interference and then measuring Nod1 mRNA levels by quantitative polymerase chain reaction (PCR). While a non-targeting small interfering RNA (siRNA control) had no effect on Nod1 mRNA levels, transfection with siRNA Nod1 decreased Nod1 mRNA levels by > 50% after 1, 2 or 3 days of transfection (Fig. 2A). Nod1-depleted HeLa cells were therefore co-transfected with the luciferase reporter plasmid, and NF-κB activation was measured after chlamydial infection. Although Nod1 depletion had no effect on NF-κB activation due to TNFα treatment (not shown), the depletion led to a partial but significant decrease in NF-κB activation following a 6 h infection with *C. muridarum* (Fig. 2B). Thus, when Nod1 activity is inhibited by overexpression of a dominant-negative Nod1 construct or Nod1 is depleted by RNA interference, there is a partial but reproducible decrease in NF-κB activation due to chlamydial infection.

**Effect of Nod1 on the transcription of pro-inflammatory genes in primary fibroblasts infected with Chlamydia**

As inhibiting or depleting Nod1 had a small effect on NF-κB activation during *Chlamydia* infection, we next evaluated whether transcription of pro-inflammatory genes is
affected by Nod1. For these experiments, murine embryonic fibroblasts (MEF) were isolated from Nod1-deficient mice and wild-type mice from the same genetic background. There was a large increase in transcription for the genes encoding the cytokine IL-6 (Fig. 3A) and the chemokine Mip-2 (Fig. 3B) after a 24 h infection of wild-type fibroblasts with C. muridarum, as measured by quantitative PCR. In contrast, there was a pronounced decrease in gene transcription in Nod1-deficient fibroblasts infected for the same length of time (Fig. 3), compared with wild-type controls, implying that Nod1 plays a role in transcription of pro-inflammatory cytokine genes during chlamydial infection.

Fig. 3. Effect of Nod1 on cytokine gene transcription during infection of fibroblasts with Chlamydia. Wild-type and Nod1-deficient MEF were infected with C. muridarum at an moi of 1.0 for 0 or 24 h, and (A) IL-6 or (B) Mip-2 gene transcription was measured by real-time PCR, as described in Experimental procedures. A representative experiment of three separate experiments is shown.

Lack of effect of Nod1 on secretion of pro-inflammatory cytokines in vaginally infected mice

To determine whether Nod1 stimulation may also contribute to the inflammatory response against Chlamydia infection in vivo, we measured the release of pro-inflammatory cytokines in the genital tract of Nod1-deficient and wild-type mice. As shown in Fig. 4, Nod1-deficient mice secreted similar amounts of the inflammatory mediators IFNγ, MIP-1α and MIP-2 (Fig. 4), and TNFα, IL-6 and IL-1β (not shown), as control wild-type mice during vaginal infection with C. muridarum. Thus, despite the role of Nod1 in transcription of pro-inflammatory cytokine genes in infected fibroblasts, the cytosolic PRR does not play a measurable role in secretion of pro-inflammatory cytokines in an animal model of genital tract infection.

No effect of Nod1 and little effect of Rip2 on the course of infection in vaginally infected mice

As cytokine secretion was similar in Nod1-deficient and wild-type mice, we also measured the time-course of the infection in both groups of mice. The percentage of infected mice was essentially the same in both groups.
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showed that the efficiency of infection was similar in both groups of mice, although lasting slightly longer in Rip2-deficient mice (Fig. 6). Rip2 is downstream from Nod1 and Nod2, but is apparently also stimulated by TLR2 and TLR4 (Chin et al., 2002; Kobayashi et al., 2002). As the infection was similar in wild-type and Nod1-deficient mice, the results obtained with Rip2-deficient mice are consistent with previous studies showing that infection is resolved with similar kinetics in Tlr2- and Tlr4-deficient mice, compared with wild-type controls (Darville et al., 2003).

Fig. 4. Lack of effect of Nod1 on chemokine and cytokine secretion during infection of mice with Chlamydia. Wild-type and Nod1-deficient mice were vaginally infected with C. muridarum, and (A) IFNγ (B) Mip-1α, and (C) Mip-2 levels in genital tract secretions were measured by ELISA, as described in Experimental procedures. Open circles, Nod1-deficient mice; closed circles, wild-type mice. A representative experiment of two separate experiments is shown.

(Fig. 5), suggesting that Nod1 is not required for mice to resolve primary infection with C. muridarum. Given the possibility that human strains may be more sensitive than C. muridarum to minor variations in the levels of cytokines such as IFNγ, the infection was also performed with C. trachomatis serovar E, which again showed no effect of Nod1 (not shown).

These results were confirmed by infecting Rip2-deficient and wild-type mice with C. muridarum, which

Fig. 5. Lack of effect of Nod1 on the duration of Chlamydia infection in mice. Wild-type and Nod1-deficient mice were vaginally infected with C. muridarum, and chlamydial infectious forming units (ifu) were measured from lower genital-tract swabs, as described in Experimental procedures. Open circles, Nod1-deficient mice; closed circles, wild-type mice. A representative experiment of two separate experiments is shown. Similar results were obtained with wild-type and Nod1-deficient mice infected with C. trachomatis serovar E.

Fig. 6. Mild effect of Rip2 on the duration of Chlamydia infection in mice. Wild-type and Rip2-deficient mice were vaginally infected with C. muridarum, and chlamydial ifu were measured from lower genital-tract swabs, as described in Experimental procedures. Open circles, Rip2-deficient mice; closed circles, wild-type mice. A representative experiment of two separate experiments is shown.
IFNγ-secreting CD4+ cells are required for Chlamydia eradication from the genital tract (Igietseme et al., 1993; Perry et al., 1997). To determine whether IFNγ-producing TH1 cells are recruited differentially to the genital tract of Rip2-deficient and wild-type mice, the mice were infected for 10 days with C. muridarum, cells from iliac lymph nodes (ILN) were collected, and lymphocytes were stained for CD4, the activation marker CD69, and intracellular IFNγ and IL-4. TH1 cells were defined as CD4+CD69+ cells that produce IFNγ in the absence of IL-4 (Maxion et al., 2004). Although TH1 cells were recruited in similar numbers (Fig. 7A), there was a slightly larger number of TH1 cells in Rip2-deficient mice, possibly reflecting the increased bacterial load in these mice. Consistent with the small effect of Rip2 on TH1 recruitment, there was also a mild elevation in oviduct inflammation in Rip2-deficient mice, as measured by oviduct dilation (Fig. 7B).

Discussion

Chlamydia trachomatis initially invades genital tract epithelial cells as well as resident myeloid cells such as macrophages (La Verda and Byrne, 1994; Rank, 1999; Morrison and Caldwell, 2002). Both infected epithelial cells and myeloid cells produce inflammatory mediators that in turn induce an influx of polymorphonuclear leukocytes and natural killer cells (Barteneva et al., 1996; Tseng and Rank, 1998). A number of pro-inflammatory mediators are secreted during infection, including IL-1, IL-6, IFNγ and TNFα, which have been detected in the fallopian tubes from humans infected with C. trachomatis and in secretions from Chlamydia-infected mice (Rank, 1999).

The inflammatory response, although harmful to the host, is indispensable for clearance of the bacteria from the infected tissue. In this study we therefore investigated one of the mechanisms by which early cytokine secretion may be regulated directly in Chlamydia-infected epithelial cells.

Previous studies have shown the importance of NF-κB in regulating cytokine and chemokine induction during bacterial infection and implicate this transcription factor as a key regulator of the inflammatory process during infection in vivo. Thus, NF-κB was activated in epithelial cells infected by bacterial pathogens such as Salmonella, Neisseria, enteropathogenic Escherichia coli or S. flexneri (Naumann, 2000; Philpott et al., 2000). Our findings confirm that Chlamydia also activates the transcription factor NF-κB. NF-κB activity was maximal at 6 h post infection and was downregulated by 24 h post infection.

However, the pathway leading to NF-κB activation by Chlamydia infection has not been defined yet. The initial recognition of pathogens is mediated by PRR that recognize PAMP. Engagement of surface TLR leads to activation of the transcription factor NF-κB and thereby production of inflammatory cytokines such as TNFα, IL-1 and IL-6 (Janeway and Medzhitov, 2002; Takeda et al., 2003). Chlamydia has several cell wall and outer membrane components that could serve as PAMP recognized by TLR. Among surface-exposed TLR, a predominant role has recently been described for TLR2 in the recognition process of C. muridarum (Darville et al., 2003), but the innate immune response to chlamydial infection was only partial dependent on TLR2 for activation of a cytokine response and the time-course of infection was unaffected by TLR2. Larger effects on clearance of infection by both C. muridarum and C. pneumoniae and the immune response were reported in mice that are deficient in the adaptor, myeloid differentiation factor 88 (MyD88) (Rothfuchs et al., 2004; Nagarajan et al., 2005; Naiki et al., 2005; Rodriguez et al., 2005), which is downstream from several TLR. These results suggested that other PRR could also detect the presence of the chlamydiae. Interestingly, the human endocervical epithelial cell line HeLa responds to C. trachomatis and to invasive strains of S. flexneri with release of the pro-inflammatory cytokine IL-8 (Rasmussen et al., 1997; Philpott et al., 2000). For S. flexneri this response is mediated by an intracellular TLR-like protein, called Nod1. We therefore investigated the role that Nod1 may play in initiation of the inflammatory response in Chlamydia-infected cells and animals.

Results obtained with cell lines and primary fibroblasts demonstrated a contribution of Nod1 in Chlamydia-induced NF-κB activation. By overexpressing a dominant-negative version of Nod1 or depletion of Nod1 by RNA interference in HeLa cells infected with C. trachomatis or...
C. muridarum, we were able to show a reproducible and statistically significant reduction in NF-κB activation during infection, compared with controls. However, the inhibition of NF-κB activation due to Nod1 inhibition or depletion was very weak. Larger effects of Nod1 were observed when we measured transcription of genes encoding pro-inflammatory mediators in wild-type and Nod1-deficient fibroblasts. Several possibilities may explain the difference between the results obtained with the HeLa cells and the fibroblasts. First of all, activation of NF-κB may not necessarily be linearly proportional to gene transcription, which could allow small differences in NF-κB activation to have large effects on gene transcription. Second, the expression of Nod1 or the cellular context in which Nod1 functions may be different in epithelial cells and fibroblasts, as recently shown for TLR (Kurt-Jones et al., 2004). Finally, the availability of chlamydial PAMP may vary as a function of cell types and perhaps tissues.

Despite the effects of Nod1 on NF-κB activation and gene transcription in vitro, Nod1 had no effect on cytokine and chemokine secretion in vaginally infected mice. Moreover, the absence of Nod1 played no role in clearance of either C. muridarum or C. trachomatis in infected mice. These results were confirmed by infecting mice that are deficient in the downstream mediator of Nod1, the kinase Rip2, as the time-course of infection was similar in wild-type and Rip2-deficient mice, although the infection may last a little longer and oviduct pathology may be exacerbated slightly in the Rip2-deficient mice. Furthermore, the results with the Rip2-deficient mice rule out the possibility that Nod2, which should recognize both Gram-positive and Gram-negative bacteria, could play a major role in the immune response to Chlamydia infection in vivo. The difference between the results obtained in vitro and in vivo underscores the important role that redundancy in the TLR system may play in the immune response of whole animals. As the prototypical surface-exposed TLR – TLR2 and TLR4 – also had no effect on the time-course of C. muridarum infection in mice (Darville et al., 2003), several surface-exposed and cytosolic PRR, or their downstream mediators, may need to be eliminated before an effect on the kinetics of chlamydial infection is observed in vivo.

Recent studies have identified a PGN motif as the PAMP for Nod1 (Chamaillard et al., 2003a; Inohara et al., 2005). Our results in vitro therefore raise the question of whether Chlamydia may produce PGN. Several biochemical studies performed in the 1960s and 1970s reported that muramic acid, a component of PGN, was present in EB, but more recent work using more reliable techniques has failed to confirm the presence of muramic acid in Chlamydia (Chopra et al., 1998; Ghuysen and Goffin, 1999). Moreover, the PGN layer is not detected in chlamydiae by electron microscopy, and antibodies against PGN do not react with chlamydiae. Nonetheless, unlike other bacteria that lack PGN, Chlamydia species have penicillin binding proteins; and penicillin, which targets biosynthesis of PGN, inhibits the developmental cycle of Chlamydia (Chopra et al., 1998; Ghuysen and Goffin, 1999). In addition, analysis of a number of Chlamydia genomes revealed that chlamydiae contain a complete set of genes for synthesizing PGN (Stephens et al., 1998; Kalman et al., 1999; Read et al., 2000; 2003). Finally, genes encoding enzymes involved in the biosynthesis of PGN, such as the murA and murC genes from C. trachomatis, are expressed in chlamydiae and the enzymes are functional when expressed in E. coli (Griffiths and Gupta, 2002; Hesse et al., 2003; McCoy et al., 2003).

Thus, it seems likely that chlamydiae may produce and require only small amounts of PGN (Chopra et al., 1998). This paradox, known as the ‘chlamydial anomaly’, is still being debated in the light of genomic information. But results from this study suggest strongly that C. muridarum and C. trachomatis produce at least the minimal PGN motif recognized by Nod1. Larger effects of Nod1 on NF-κB activation were recently observed with endothelial cells infected in vitro with C. pneumoniae (Opitz et al., 2005), suggesting that all Chlamydiaceae may produce at least the rudiments of a functional PGN. Further studies should elucidate how chlamydial PGN, produced in the chlamydial vacuoles, can cross the inclusion membrane to enter the host-cell cytosol.

Experimental procedures

Cells, animals and materials

The human cervical adenocarcinoma cell line, HeLa229, was from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in a humidified incubator at 37°C with 5% CO₂ in Dulbecco’s modified minimal essential medium (DMEM) with Glutamax-1 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 25 μg/ml 10⁻³ gentamicin. The MoPn agent of C. trachomatis (Nigg, also known as C. muridarum), the C. trachomatis LGV2 strain, and C. trachomatis serovar E were obtained originally from ATCC. The chlamydiae were grown in McCoy cells and purified as previously described (Ramsey et al., 1989). The human recombinant cytokine TNFα was purchased from Sigma (St Louis, MO). Other cell culture reagents were previously described (Coutinho-Silva et al., 2003; Darville et al., 2003; Perfettini et al., 2003).

In order to maintain a colony of receptor or kinase-deficient mice, Nod1⁻ mice were bred with Nod1⁻ females (Girardin et al., 2003b) and Rip2⁻ mice with Rip2⁻ females (Chin et al., 2002). Genetically comparable wild-type mice (C57BL/6) were used as controls in experiments with both Nod1⁻ and Rip2-deficient mice. MEF were isolated from Nod1⁻ and wild-type mice following established procedures (Wei et al., 2001).
Transient transfection of cells and infection with Chlamydia

For experiments with dominant-negative Nod1, HeLa cells were first seeded at 2 × 10^5 on 24 well plates (Costar, New York, NY) 24 h before transfection with Lipofectamine and Plus transfection reagents (Invitrogen), according to the manufacturer's instructions. Each sample contained 400 ng of a NF-κB-luciferase reporter plasmid (Ten et al., 1992; Munoz et al., 1994) and 50–300 ng of vector expressing the dominant-negative form of Nod1 (Girardin et al., 2001). The empty plasmid pcDNA3 (Invitrogen, Carlsbad, CA) was used to maintain the total amount of plasmid constant at 700 ng. After 24 h of transfection, cells were left untreated or treated for 6 h at 37°C with 100 ng ml⁻¹ TNFα, or infected with C. muridarum or C. trachomatis LGV2 at a multiplicity of infection (moi) of 1.0 for 6 h at 37°C, as described (Perfettini et al., 2003). Alternatively, cells that had been transfected with the NF-κB-luciferase reporter plasmid but without the dominant-negative Nod1 constructs were infected with C. muridarum or C. trachomatis LGV2 at an moi of 1.0 for the indicated periods of time. Luciferase activity was measured in the cell lysates as previously described (Philpott et al., 2000), using an EGNG Berthold luminometer (Thoiry, France). Results are expressed as relative luciferase units.

Measurement of gene transcription by real-time PCR

Murine embryonic fibroblast were infected with C. muridarum at an moi of 1.0 for 24 h, as previously described for lung fibroblasts (Darville et al., 2003). RNA was prepared and reverse-transcribed from uninfected and infected fibroblasts, as described (Darville et al., 2003). Quantitative PCR was performed with 1/50 of the cDNA preparation in the Mx3000P (Stratagene) in 25 µl final volumes with the Brilliant SYBR Green QPCR Master Mix (Stratagene). The specificity of the PCR was confirmed by melting curve analysis at each data point. The primers used for PCR had the sequences in Table 2.

SMARTpool reagents for human CARD4 (siRNA Nod1) were purchased from Dharmacon (Perbio, France). In order to increase the probability that siRNA might reduce Nod1 mRNA to low levels, four of Dharmacon's SMART-design-selected siRNA Nod1 were combined into a single pool. HeLa cells that had been plated at a density of 1 × 10⁴ cells on 96 well plates were transfected 1 day later with siRNAs (100 nM) using TransIT- siQuest reagent (Mirus), following the manufacturer's protocol.

For quantification experiments, RNA was prepared and reverse-transcribed from siRNA-transfected HeLa cells, as described (Darville et al., 2003). Quantitative PCR was performed in the Mx3000P (Stratagene) in 25 µl final volumes with the FullVelocity SYBR Green QPCR Master Mix (Stratagene). The specificity of the PCR was confirmed by melting curve analysis at each data point. The primers used for PCR had the sequences in Table 2.

Table 1. Primers for measurement of gene transcription in murine fibroblasts.

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<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
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<td>5'-GAATTGAAGCACGTTTCCCTCCC-3'</td>
<td>5'-FAM-CACTGCGCATCTTCTCCATCC-3'</td>
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<tr>
<td>IL-6</td>
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<td>5'-TGATTGGATGTCTGTTGCTCCT-3'</td>
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<tr>
<td>MIP-2</td>
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<td>5'-CTTGAGAGTCGGCATGACTTCTG-3'</td>
<td>5'-FAM-TGACGCCCCAGAGC-3'</td>
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Table 2. Primers for measurement of Nod1 gene transcription in HeLa cells.

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</tr>
<tr>
<td>Nod1</td>
<td>5'-CAAGTCTCACCACATCCAGAGC-3'</td>
<td>5'-AATTTTGCGGACCTTGTCAG-3'</td>
</tr>
</tbody>
</table>

Infection of mice with Chlamydia

Groups of five wild-type and five Nod1⁻⁻ mice, or five wild-type and five Rip2⁻⁻ mice, received 2.5 mg of medroxyprogesterone acetate (Depo-Provera; Upjohn, Kalamazoo, MI) subcutaneously 7 days before vaginal infection. The mice were infected by placing 30 µl of 250 µM sucrose–10 mM sodium phosphate–5 mM L-glutamic acid (SPG) containing 1.0 × 10⁷ inclusion-forming units (ifu) of C. muridarum or C. trachomatis serovar E per ml into the vagina of each mouse or 30 µl of SPG without bacteria for negative controls, as described elsewhere (Darville et al., 2001).

Table 2. Primers for measurement of Nod1 gene transcription in HeLa cells.
Measurement of chlamydial shedding

The course of infection was assessed by the culture of chlamydiae from cervico-vaginal swabs using standard isolation procedures, as previously described (Darville et al., 1997). Briefly, chlamydiae were isolated from swabs in tissue culture according to standard methods, and inclusions were visualized and enumerated by immunofluorescence (Ramsey et al., 1989). Results are expressed as mean and SE of ifu per ml. Experiments were repeated twice for infection of Nod1−/− and Rip2−/− mice.

Collection of genital tract secretions for cytokine analysis

Genital tract secretions were collected from mice on multiple days throughout the course of infection and analysed by enzyme-linked immunosorbent assay (ELISA) for IFNγ, Mip-2, TNFα, IL-6, IL-1β and Mip-1α using ELISA kits from R and D Systems (Minneapolis, MN). At intervals before and after infection, an aseptic surgical sponge (ear wicks, 2 by 5 mm) (DeRoyal; Powell, TN) was inserted into the vagina of an anaesthetized mouse and retrieved 30 min later. Samples were eluted from the sponges and analysed in an ELISA plate reader, as described elsewhere (Darville et al., 2003).

Isolation of leukocytes and characterization of TH_1 response

Iliac lymph nodes from wild-type and Rip2-deficient mice infected with C. muridarum (1.5 × 10^5 ifu per mouse) were isolated after 10 days of infection, as previously described (Ojcius et al., 1998). Single-cell leukocyte suspensions were prepared and stained for the mouse cell surface markers CD4 and CD69, and intracellular IFNγ and IL-4, as described (Maxion et al., 2004). Flow cytometry was performed with a FACSscan (Becton Dickinson, San Jose, CA).

Analysis of oviduct dilation by histopathology

For histological analysis of oviduct tissue, groups of three to eight mice were sacrificed after 49 days of infection with C. muridarum (1.5 × 10^5 ifu per mouse). The upper genital tract was removed, and a latitudinal incision was made (Maxion et al., 2004). Individual oviducts were submerged in Optimal Cutting Temperature embedding medium (Tissue Tek; Sâkura Finetek, Torance, CA) and stored at −80°C. Frozen tissue blocks were cut transversally from the ovary and oviduct, and sections were collected at the beginning of the transitional region between ovary and oviduct. Sections were stained with haematoxylin and eosin (H&E), and the diameter of the oviduct lumen was measured using a grid-containing lens (×10) (BH-2; Olympus, Tokyo, Japan). Each value is a mean of four diameter measurements for a single oviduct (Maxion et al., 2004).

Statistics

Statistical comparisons between the groups of mice for level of infection and cytokine or chemokine production over the course of infection were made by a two-factor (days and murine strain) analysis of variance with the post hoc Turkey test as a multiple-comparison procedure. The Wilcoxon rank sum test was used to compare the duration of infection in the respective groups of mice. The effects of C. muridarum or C. trachomatis during infection of cell lines and inhibitory effects of dominant-negative constructions were statistically evaluated using the Student’s t-test.

Acknowledgements

This work was supported by the National Institutes of Health Grant R01 AI054624, Université Paris – Denis Diderot, and the University of California. We thank Thomas Juntas, Paul Dempsey and Jim Sikes for excellent technical assistance, and Dr Philippe Verbeke (Université Paris – Denis Diderot) for valuable discussions.

References


