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-κB 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-κB 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 multiplicative 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$^+$ TH$_1$ 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; O'Neill, 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 I-κB kinase complex (IKK), which phosphorylates I-κB, 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 diaminopimelate (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; Ghuysen 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.

Fig. 1. Effect of a Nod1 dominant-negative construct on NF-κB activation during infection of epithelial cells with *Chlamydia*. HeLa cells were co-transfected with a NF-κB-luciferase reporter plasmid and increasing concentrations of a dominant-negative (DN) Nod1 construct or control vector, and then infected with *C. muridarum* at an moi of 1.0 for 6 h, as described in *Experimental procedures*. Luciferase activity was expressed as relative NF-κB activity. Basal levels of NF-κB activation (no infection) were subtracted from all the conditions. $P < 0.05$ for 100 ng and 200 ng Nod1 DN, compared with vector alone (0 ng Nod1 DN at 6 h of infection). The time points were performed in triplicate, and a representative experiment of three separate experiments is shown.

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 oviduct 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 concen-
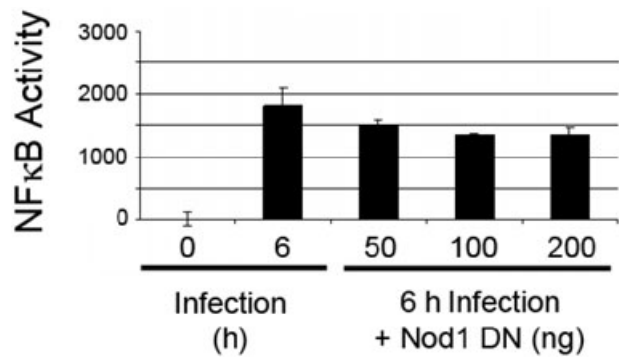
trations, there was essentially no effect of transfection on NF-κB activation due to tumour necrosis factor (TNFα) treatment (not shown), in agreement with previous studies (Girardin *et al.*, 2001).

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
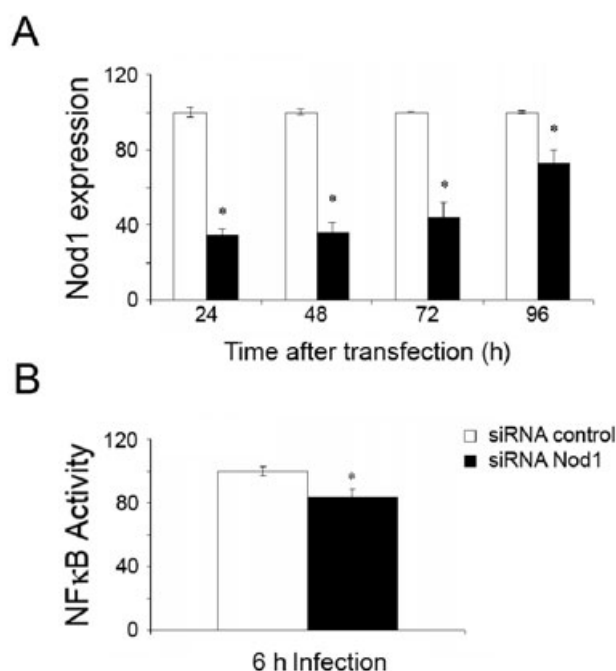
A

B

Fig. 2. Effect of Nod1 depletion on NF-κB activation during infection of epithelial cells with *Chlamydia*. HeLa cells were depleted of Nod1 by RNA interference (siRNA Nod1) or transfected with an irrelevant control (siRNA control), as described in *Experimental procedures*.
A. On indicated times after siRNA treatment, Nod1 mRNA was measured by real-time PCR, using primers and conditions described in *Experimental procedures*. $P < 0.05$ for siRNA Nod1, compared with siRNA control for all transfection times.
B. Cells transfected with siRNA control or siRNA Nod1 for 3 days were then transfected with NF-κB-luciferase reporter plasmid, as described in *Experimental procedures*. After an additional 24 h incubation, the co-transfected cells were infected with *C. muridarum* at an moi of 1.0 for 6 h. Luciferase activity was expressed as relative NF-κB activity. $P < 0.01$ for siRNA Nod1, compared with siRNA control (6 h of infection). The time points were performed in triplicate, and a representative experiment of three separate experiments is shown.

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.

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

To determine whether Nod1 stimulation may also contrib-

ute 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
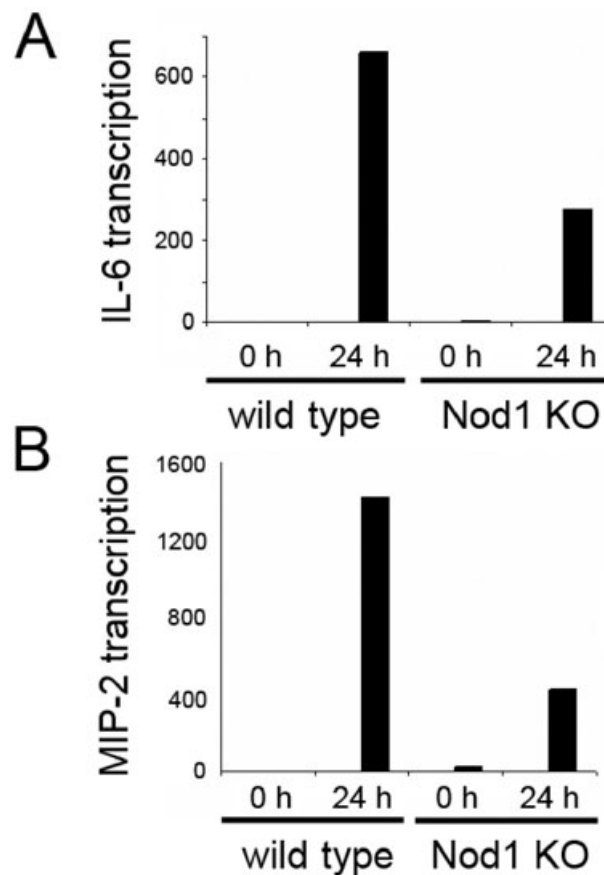
A

B

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.
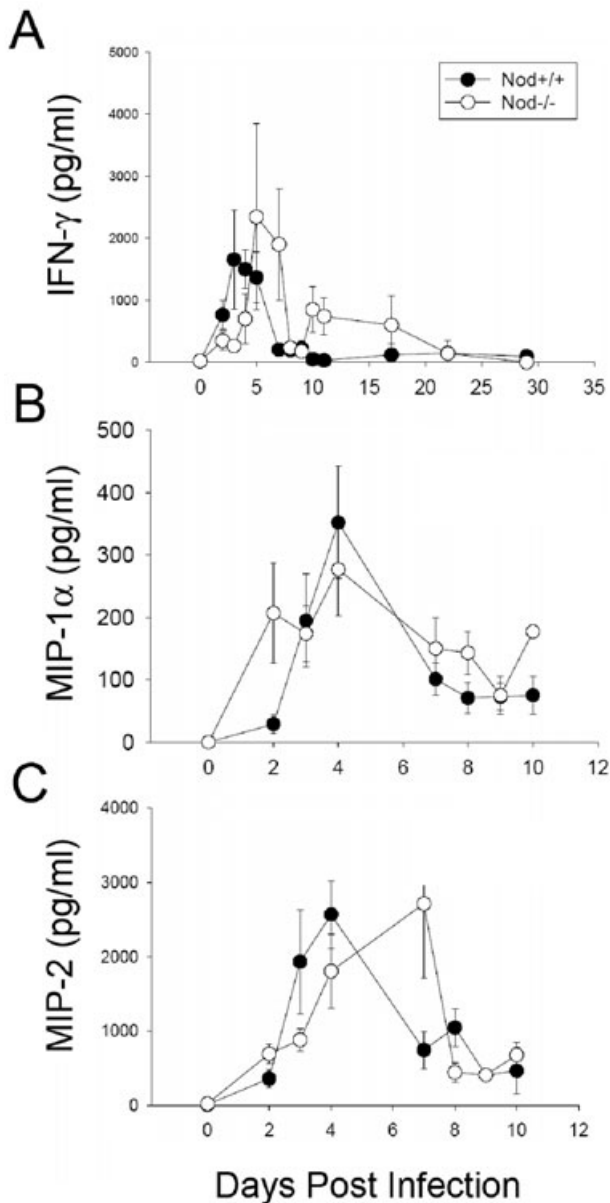
Fig. 4. Lack of effect of Nod1 on chemokine and cytokine secretion during infection of mice with *Chlamydia*. Wild-type and *Nod1*-deficient mice were vaginally infected with *C. muridarum*, and (A) IFNγ (B) Mip-1α, and (C) Mip-2 levels in genital tract secretions were measured by ELISA, as described 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

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).
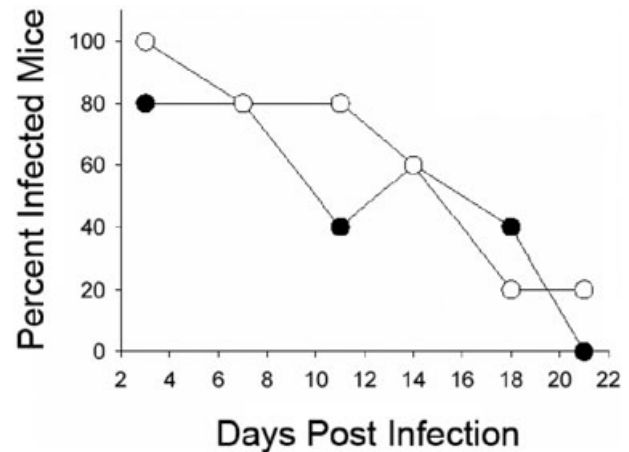

Fig. 5. Lack of effect of Nod1 on the duration of *Chlamydia* infection in mice. Wild-type and *Nod1*-deficient mice were vaginally infected with *C. muridarum*, and chlamydial infectious forming units (ifu) were measured from lower genital-tract swabs, as described in *Experimental procedures*. Open 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.
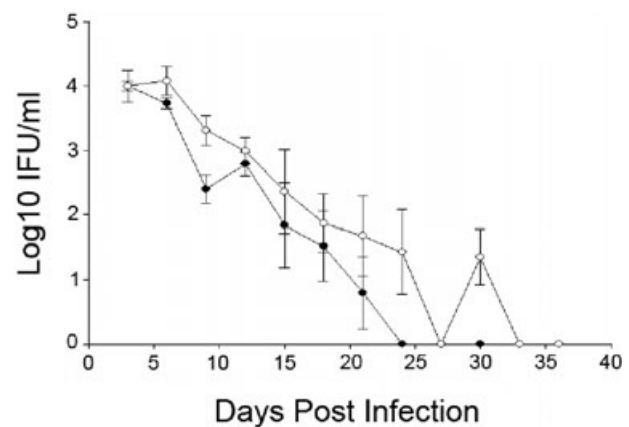

Fig. 6. Mild effect of Rip2 on the duration of *Chlamydia* infection in mice. Wild-type and *Rip2*-deficient mice were vaginally infected with *C. muridarum*, and chlamydial ifu were measured from lower genital-tract swabs, as described in *Experimental procedures*. Open circles, *Rip2*-deficient mice; 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 TH$_1$ 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. TH$_1$ cells were defined as CD4^{+}CD69^{+} cells that produce IFNγ in the absence of IL-4 (Maxion *et al.*, 2004). Although TH$_1$ cells were recruited in similar numbers (Fig. 7A), there was a slightly larger number of TH$_1$ cells in *Rip2*-deficient mice, possibly reflecting the increased bacterial load in these mice. Consistent with the small effect of Rip2 on TH$_1$ 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 leucocytes 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 fallo-
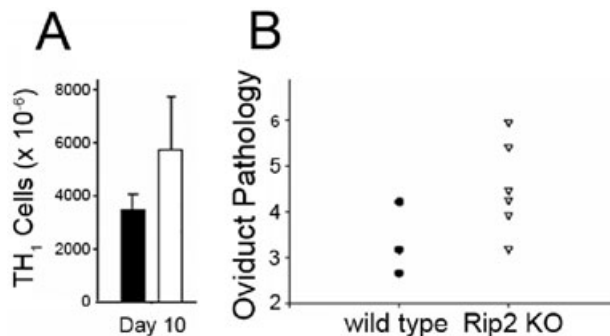

Fig. 7. Mild effect of Rip2 on the immune response and pathology in *Chlamydia*-infected mice.
A. Number of IFNγ-producing CD4^{+} (TH1) cells in ILN tissues per 10[6] lymphocytes in wild-type mice (black bar) and *Rip2*-deficient mice (white bar) 10 days after infection with *C. muridarum*. Cells were isolated and stained as described in *Experimental procedures*. Data were compiled from two independent experiments. $P = 0.311$ for *Rip2*-deficient mice, compared with wild-type mice.
B. Oviduct dilation in wild-type and *Rip2*-deficient mice after a 49 day infection with *C. muridarum*. Oviduct diameters were measured from H&E-stained sections collected transversally at the ovary-to-oviduct transition of mice from two independent experiments. Data are expressed as the average number of grids (one grid ~ 1 μm) of two diameter measurements per oviduct section of four sections per oviduct. $P = 0.119$ for *Rip2*-deficient mice, compared with wild-type mice.

pian 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 depleting 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% CO_2 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^{-1} 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*$^{-/-}$ males were bred with *Nod1*$^{-/-}$ females (Girardin *et al.*, 2003b) and *Rip2*$^{-/-}$ males 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).

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

Gene	Forward primer	Reverse primer	Probe
β-Actin	5′-AGAGGGAAATCGTGCGTGAC-3′	5′-CAATAGTGATGACCTGGCCGT-3′	5′-FAM-CACTGCCGCATCCTCTTCCTCCC-TAMRA-3′
IL-6	5′-TCCTACCCCAATTTCCAATGC-3′	5′-TGAATTGGATGGTCTTGGTCCT-3′	5′-FAM-CAGATAAGCTGGAGTCACAGAAGGAGTGG-TAMRA-3′
MIP-2	5′-TGACTTCAAGAACATCCAGAGCTT-3′	5′-CTTGAGAGTGGCTATGACTTCTGTCT-3′	5′-FAM-TGACGCCCCCAGGACCCCA-TAMRA-3′

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 QPCR Master Mix (Stratagene). cDNA was amplified using 300 nM of each specific sense and antisense primers. We also used 100 nM of the fluorogenic oligonucleotides specific for the gene segments in which a reporter fluorescent dye on 5′ (FAM) and a quencher dye on 3′ (TAMRA) were attached. Quantitative PCR was conducted at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min and 72°C for 30 s. The expression levels of cytokine cDNA were compared with β-actin and normalized to uninfected wild-type fibroblast responses by the comparative cycle threshold method, as described by the manufacturer (Stratagene). The forward primers, reverse primers, and fluorescent probes for murine β-actin, IL-6 and MIP-2 are listed in Table 1.

Nod1-depletion experiments with RNA interference

The non-targeting siRNA, siRNA control, and siGENOME 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-selection-designed siRNA Nod1 were combined into a single pool. HeLa cells that had been plated at a density of 1×10^4 cells on 96 well plates were transfected 1 day later with siRNAs (100 nM) using *Trans*IT-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.

cDNA was amplified using 300 nM of Gapdh forward- and reverse-primers and 300 nM of Nod1 forward primer and 150 nM of Nod1 reverse primer. Cycling conditions were as follows: at 95°C for 5 min, then 40 cycles at 95°C for 10 s and 60°C for 30 s.

For luciferase reporter essays, the cells that had been transfected with siRNA control or siRNA Nod1 were transfected 3 days later with NF-κB-luciferase plasmid, were incubated for 24 h, and then were infected for 6 h with *C. muridarum* or, as control, treated with 100 ng ml⁻¹ TNFα (Girardin *et al.*, 2001) before analysis.

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 mM sucrose–10 mM sodium phosphate–5 mM L-glutamic acid (SPG) containing 1.0×10^7 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.

Gene	Forward primer	Reverse primer
Gapdh	5′-GAGTCAACGGATTTGGTCGT-3′	5′-TTGATTTTGGAGGGATCTCG-3′
Nod1	5′-CAGAGTCTCACCCCCACATT-3′	5′-AATTTTGCGGACCTTGTCAG-3′

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 FACScan (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; Sakura Finetek, Torance, CA) and stored at $-80°$C. Frozen tissue blocks were cut transversally from the ovary, 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 ($\times$ 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.

References

Bäckhed, F., and Hornef, M. (2003) Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? *Microbes Infect* **5:** 951–959.

Barron, A.L., White, H.J., Rank, R.G., Soloff, B.L., and Moses, E.B. (1981) A new animal model for the study of *Chlamydia trachomatis* genital infections: infection of mice with the agent of mouse pneumonitis. *J Infect Dis* **143:** 63–66.

Barteneva, N., Theodor, I., Peterson, E.M., and de la Maza, L.M. (1996) Role of neutrophils in controlling early stages of a *Chlamydia trachomatis* infection. *Infect Immun* **64:** 4830–4833.

Belland, R.J., Zhong, G., Crane, D.D., Hogan, D., Sturdevant, D., Sharma, J., *et al.* (2003) Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. *Proc Natl Acad Sci USA* **100:** 8478–8483.

Belland, R., Ojcius, D.M., and Byrne, G.I. (2004) Chlamydia. *Nat Rev Microbiol* **2:** 530–531.

Bertin, J., Nir, W.J., Fischer, C.M., Tayber, O.V., Errada, P.R., Grant, J.R., *et al.* (1999) Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-κB. *J Biol Chem* **274:** 12955–12958.

Boule, M.W., Broughton, C., Mackay, F., Akira, S., Marshak-Rothstein, A., and Rifkin, I.R. (2004) Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J Exp Med* **199:** 1631–1640.

Cain, T.K., and Rank, R.G. (1995) Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis (MoPn) biovar of *Chlamydia trachomatis*. *Infect Immun* **63:** 1784–1789.

Cario, E., Rosenberg, I.M., Brandwein, S.L., Beck, P.L., Reinecker, H.C., and Podolsky, D.K. (2000) Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J Immunol* **164:** 966–972.

Chamaillard, M., Girardin, S.E., Viala, J., and Philpott, D.J. (2003a) Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell Microbiol* **5:** 581–592.

Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Su, Q., Saab, L., *et al.* (2003b) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* **4:** 702–707.

Chin, A.I., Dempsey, P.W., Bruhn, K., Miller, J.F., Xu, Y., and Cheng, G.H. (2002) Involvement of receptor-interacting

protein 2 in innate and adaptive immune responses. *Nature* **416:** 190–194.

Chopra, I., Storey, C.C., Falla, T.J., and Pearce, J.H. (1998) Antibiotics, peptidoglycan synthesis and genomics: the chlamydial anomaly revisited. *Microbiology* **144:** 2673–2678.

Coutinho-Silva, R., Stahl, L., Raymond, M.N., Jungas, T., Verbeke, P., Burnstock, G., *et al.* (2003) Inhibition of chlamydial infectious activity due to P2X$_7$ receptor-dependent phospholipase D activation. *Immunity* **19:** 403–412.

Darville, T., Andrews, C.W., Laffoon, K.K., Shymasani, W., Kishen, L.R., and Rank, R.G. (1997) Mouse strain-dependent variation in the course and outcome of chlamydial genital infection is associated with differences in host response. *Infect Immun* **65:** 3064–3073.

Darville, T., Andrews, C.W., Sikes, J.D., Fraley, P.L., and Rank, R.G. (2001) Early local cytokine profiles in strains of mice with different outcomes from chlamydial genital tract infection. *Infect Immun* **69:** 3556–3561.

Darville, T., O'Neill, J.M., Andrews, C.W., Jr, Nagarajan, U.M., Stahl, L., and Ojcius, D.M. (2003) Toll-like receptor-2, but not Toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection. *J Immunol* **171:** 6187–6197.

Derbigny, W.A., Kerr, M.S., and Johnson, R.M. (2005) Pattern recognition molecules activated by *Chlamydia muridarum* infection of cloned murine oviduct epithelial cell lines. *J Immunol* **175:** 6065–6075.

Everett, K.D.E., Bush, R.M., and Andersen, A.A. (1999) Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* **49:** 415–440.

Gerbase, A.C., Rowley, J.T., and Mertens, T.E. (1998) Global epidemiology of sexually transmitted diseases. *Lancet* **351:** 2–4.

Ghuysen, J.M., and Goffin, C. (1999) Lack of cell wall peptidoglycan versus penicillin sensitivity: new insights into the chlamydial anomaly. *Antimicrob Agents Chemother* **43:** 2339–2344.

Girardin, S.E., Tournebize, R., Mavris, M., Page, A.L., Li, X., Stark, G.R., *et al.* (2001) CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive *Shigella flexneri*. *EMBO Rep* **2:** 736–742.

Girardin, S.E., Sansonetti, P., and Phillpott, D.J. (2002) Intracellular vs extracellular recognition of pathogens – common concepts in mammals and flies. *Trends Microbiol* **10:** 193–199.

Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., *et al.* (2003a) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* **278:** 8869–8872.

Girardin, S.E., Boneca, I.G., Carneiro, L.A.M., Antignac, A., Jéhanno, M., Viala, J., *et al.* (2003b) Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* **300:** 1584–1587.

Griffiths, E., and Gupta, R.S. (2002) Protein signatures distinctive of chlamydial species: horizontal transfers of cell wall biosynthesis genes *glmU* from archaea to chlamydiae and *murA* beetween chlamydiae and *Streptomyces*. *Microbiology* **148:** 2541–2549.

Hackstadt, T. (1999) Cell biology. In Chlamydia: *Intracellular Biology, Pathogenesis, and Immunity.* Stephens, R.S. (ed.). Washington, DC: American Society for Microbiology Press, pp. 101–138.

Hesse, L., Bostock, J., Dementin, S., Blanot, D., Mengin-Lecreulx, D., and Chopra, I. (2003) Functional and biochemical analysis of *Chlamydia trachomatis* MurC, an enzyme displaying UDP-N-acetylmuramate: amino acid ligase activity. *J Bacteriol* **185:** 6507–6512.

Igietseme, J.U., Ramsey, K.H., Magee, D.M., Williams, D.M., Kincy, T.J., and Rank, R.G. (1993) Resolution of murine chlamydial genital infection by the adoptive transfer of a biovar-specific, Th1 lymphocyte clone. *Reg Immunol* **5:** 317–324.

Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., *et al.* (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem* **274:** 14560–14567.

Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., *et al.* (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem* **278:** 5509–5512.

Inohara, N., Chamaillard, M., McDonald, C., and Nunez, G. (2005) NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem* **74:** 355–383.

Janeway,C.A., Jr and Medzhitov, R. (2002) Innate immune recognition. *Annu Rev Immunol* **20:** 197–216.

Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R.W., *et al.* (1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* **21:** 385–389.

Karikó, K., Ni, H., John Capodici, J., Lamphier, M., and Weissman, D. (2004) mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem* **279:** 12542–12550.

Kobayashi, K., Inohara, N., Hernandez, L.D., Galan, J.E., Nuñez, G., Janeway, C., *et al.* (2002) RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* **416:** 194–199.

Kurt-Jones, E.A., Sandor, F., Ortiz, Y., Bowen, G.N., Counter, S.L., Wang, T.C., and Finberg, R.W. (2004) Use of murine embryonic fibroblasts to define Toll-like receptor activation and specificity. *J Endotoxin Res* **10:** 419–424.

La Verda, D., and Byrne, G.I. (1994) Interactions between macrophages and chlamydiae. *Immunol Series* **60:** 381–399.

McCoy, A.J., Sandlin, R.C., and Maurelli, A.T. (2003) *In vitro* and *in vivo* functional activity of *Chlamydia MurA*, a UDP-N-acetylglucosamine enolpyruvyl transferase involved in peptidoglycan synthesis and fosfomycin resistance. *J Bacteriol* **185:** 1218–1228.

Maxion, H.K., Liu, W., Chang, M.-H., and Kelly, K.A. (2004) The infecting dose of *Chlamydia muridarum* modulates the innate immune response and ascending infection. *Infect Immun* **72:** 6330–6340.

Medzhitov, R., and Janeway, C.A., Jr (2002) Decoding the patterns of self and nonself by the innate immune system. *Science* **296:** 298–300.

Morrison, R.P., and Caldwell, H.D. (2002) Immunity to murine chlamydial genital infection. *Infect Immun* **70:** 2741–2751.

Moulder, J.W. (1991) Interaction of chlamydiae and host cells *in vitro*. *Microbiol Rev* **55:** 143–190.

Munoz, G., Courtois, P., Veschambre, P., Jalinot, P., and Israel, A. (1994) Tax induces nuclear translocation of NF-kappa B through dissociation of cytoplasmic complexes

containing p105 or p100 but does not induce degradation of I kappa B alpha/MAD3. *J Virol* **68:** 8035–8044.

Nagarajan, U.M., Ojcius, D.M., Stahl, L., Rank, R.G., and Darville, T. (2005) *Chlamydia trachomatis* induces expression of IFN-gamma-inducible protein 10 and IFN-beta independent of TLR2 and TLR4, but largely dependent on MyD88. *J Immunol* **175:** 450–460.

Naiki, Y., Michelsen, K.S., Schroder, N.W., Alsabeh, R., Slepenkin, A., Zhang, W., *et al.* (2005) MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance and survival in a mouse model of *Chlamydia pneumoniae* pneumonia. *J Biol Chem* **280:** 29242–29249.

Naumann, M. (2000) Nuclear factor-kappa B activation and innate immune response in microbial pathogen infection. *Biochem Pharmacol* **60:** 1109–1114.

Nicholson, T.L., Olinger, L., Chong, K., Schoolnik, G., and Stephens, R.S. (2003) Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*. *J Bacteriol* **185:** 3179–3189.

Nishimura, M., and Naito, S. (2005) Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. *Biol Pharm Bull* **28:** 886–892.

O'Neill, L.A. (2004) After the Toll rush. *Science* **303:** 1481–1482.

Ojcius, D.M., Bravo de Alba, Y., Kanellopoulos, J.M., Hawkins, R.A., Kelly, K.A., Rank, R.G., and Dautry-Varsat, A. (1998) Internalization of *Chlamydia* by dendritic cells and stimulation of *Chlamydia*-specific T cells. *J Immunol* **160:** 1297–1303.

Opitz, B., Forster, S., Hocke, A.C., Maass, M., Schmeck, B., Hippenstiel, S., *et al.* (2005) Nod1-mediated endothelial cell activation by *Chlamydophila pneumoniae*. *Circ Res* **96:** 319–326.

Perfettini, J.L., Ojcius, D.M., Andrews, C.W., Korsmeyer, S.J., Rank, R.G., and Darville, T. (2003) Role of proapoptotic BAX in propagation of *Chlamydia muridarum* (the mouse pneumonitis strain of *Chlamydia trachomatis*) and the host inflammatory response. *J Biol Chem* **278:** 9496–9502.

Perry, L.L., Feilzer, K., and Caldwell, H.D. (1997) Immunity to *Chlamydia trachomatis* is mediated by T helper 1 cells through IFNγ-dependent and -independent pathways. *J Immunol* **158:** 3344–3352.

Philpott, D.J., Yamaoka, S., Israel, A., and Sansonetti, P.J. (2000) Invasive *Shigella flexneri* activates NF-κB through a lipopolysaccharide-dependent innate intracellular response and leads to IL-8 expression in epithelial cells. *J Immunol* **165:** 903–914.

Ramsey, K.H., Newhall, W.J., and Rank, R.G. (1989) Humoral immune response to chlamydial genital infection of mice with the agent of mouse pneumonitis. *Infect Immun* **57:** 2441–2446.

Rank, R.G. (1999) Models of immunity. In Chlamydia: *Intracellular Biology, Pathogenesis, and Immunity.* Stephens, R.S. (ed.). Washington, DC: American Society for Microbiology Press, pp. 239–295.

Rank, R.G., Ramsey, K.H., Pack, E.A., and Williams, D.M. (1992) Effect of gamma interferon on resolution of murine chlamydial genital infection. *Infect Immun* **60:** 4427–4429.

Rasmussen, S.J., Eckmann, L., Quayle, A.J., Shen, L., Zhang, Y.X., Anderson, D.J., *et al.* (1997) Secretion of proinflammatory cytokines by epithelial cells in response to *Chlamydia* infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J Clin Invest* **99:** 77–87.

Read, T.D., Brunham, R.C., Shen, C., Gill, S.R., Heidelberg, J.F., White, O., *et al.* (2000) Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res* **28:** 1397–1406.

Read, T.D., Myers, G.S., Brunham, R.C., Nelson, W.C., Paulsen, I.T., Heidelberg, J., *et al.* (2003) Genome sequence of *Chlamydophila caviae* (*Chlamydia psittaci* GPIC): examining the role of niche-specific genes in the evolution of the Chlamydiaceae. *Nucleic Acid Res* **31:** 2134–2147.

Rodriguez, N., Fend, F., Jennen, L., Schiemann, M., Wantia, N., Prazeres da Costa, C.U., *et al.* (2005) Polymorphonuclear neutrophils improve replication of *Chlamydia pneumoniae in vivo* upon MyD88-dependent attraction. *J Immunol* **174:** 4836–4844.

Rothfuchs, A.G., Trumstedt, C., Wigzell, H., and Rottenberg, M.E. (2004) Intracellular bacterial infection-induced IFN-gamma is critically but not solely dependent on Toll-like receptor 4-myeloid differentiation factor 88-IFN-alpha beta-STAT1 signaling. *J Immunol* **172:** 6345–6353.

Schachter, J., Stephens, R.S., Timms, P., Kuo, C., Bavoil, P.M., Birkelund, S., *et al.* (2001) Radical changes to chlamydial taxonomy are not necessary just yet. *Int J Syst Evol Microbiol* **51:** 249.

Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., *et al.* (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **23:** 638–639.

Takeda, K., Kaisho, T., and Akira, S. (2003) Toll-like receptors. *Annu Rev Immunol* **21:** 335–376.

Ten, R.M., Paya, C.V., Israel, N., Le Bail, O., Mattei, M.G., Virelizier, J.L., *et al.* (1992) The characterization of the promoter of the gene encoding the p50 subunit of NF-kappa B indicates that it participates in its own regulation. *EMBO J* **11:** 195–203.

Thylefors, B., Negrel, A.D., Pararajasegaram, R., and Dadzie, K.Y. (1995) Global data on blindness. *Bull World Health Organ* **73:** 115–121.

Tseng, C.K., and Rank, R.G. (1998) Role of NK cells in the early host response to chlamydial genital infection. *Infect Immun* **66:** 5867–5875.

Ward, M.E. (1999) Mechanisms of *Chlamydia*-induced disease. In Chlamydia: *Intracellular Biology, Pathogenesis, and Immunity.* Stephens, R.S. (ed.). Washington, DC: American Society for Microbiology Press, pp. 171–210.

Wei, M.C., Zong, W.-X., Cheng, E.H.-Y., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., *et al.* (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **2:** 727–730.

Wyrick, P.B. (2000) Intracellular survival by *Chlamydia*. *Cell Microbiol* **2:** 275–282.