Cell Host & Microbe
Article

Penicillin Resistance Compromises Nod1-Dependent Proinflammatory Activity and Virulence Fitness of *Neisseria meningitidis*

Maria Leticia Zarantonelli,[1,13,17] Anna Skoczynska,[1,9,17] Aude Antignac,[1,2,17] Meriem El Ghachi,[3,10] Ala-Eddine Deghmane,[2] Marek Szatanik,[1,2] Céline Mulet,[4,11] Catherine Werts,[3,10] Lucie Peduto,[5,12] Martine Fanton d'Andon,[3,10] Françoise Thouron,[6] Faridabano Nato,[7] Lionel LeBourhis,[8,14] Dana J. Philpott,[8,15] Stephen E. Girardin,[4,11,16] Francina Langa Vives,[6] Philippe Sansonetti,[4,11] Gérard Eberl,[5,12] Thierry Pedron,[4,11] Muhamed-Kheir Taha,[1,2,*] and Ivo G. Boneca[3,10,*]
[1]Institut Pasteur, Unité des Neisseria
[2]Institut Pasteur, Unité infections bactériennes invasives
[3]Institut Pasteur, Unité Biologie et génétique de la paroi bactérienne
[4]Institut Pasteur, Unité de Pathogénie Microbienne Moléculaire
[5]Institut Pasteur, Lymphoid Tissue Development Unit
[6]Institut Pasteur, Plate-Forme Technologique Centre d'Ingénierie Génétique Murine
[7]Institut Pasteur, Plate-forme 5 Production de proteins
[8]Institut Pasteur, Groupe Immunité Innée et Signalisation
25-28 rue du Dr. Roux, Paris 75724, France
[9]National Medicines Institute, National Reference Center for Bacterial Meningitis, Warsaw 00-725, Poland
[10]INSERM, Groupe Avenir, Paris 75015, France
[11]INSERM, U389, Paris 75015, France
[12]CNRS, URA1961, Paris 75015, France
[13]Present address: Laboratorio de Biofármacos: Control & Desarrollo, Institut Pasteur de Montevideo, Montevideo CP 11400, Uruguay
[14]Present address: Institut Curie, Paris 75005, France
[15]Present address: Department of Immunology, University of Toronto, Toronto, ON M5S 1A8, Canada
[16]Present address: Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON M5S 1A8, Canada
[17]These authors contributed equally to this work
*Correspondence: mktaha@pasteur.fr (M.-K.T.), bonecai@pasteur.fr (I.G.B.)
http://dx.doi.org/10.1016/j.chom.2013.04.016

SUMMARY

Neisseria meningitidis is a life-threatening human bacterial pathogen responsible for pneumonia, sepsis, and meningitis. Meningococcal strains with reduced susceptibility to penicillin G (Pen^I) carry a mutated penicillin-binding protein (PBP2) resulting in a modified peptidoglycan structure. Despite their antibiotic resistance, Pen^I strains have failed to expand clonally. We analyzed the biological consequences of PBP2 alteration among clinical meningococcal strains and found that peptidoglycan modifications of the Pen^I strain resulted in diminished in vitro Nod1-dependent proinflammatory activity. In an influenza virus-meningococcal sequential mouse model mimicking human disease, wild-type meningococci induced a Nod1-dependent inflammatory response, colonizing the lungs and surviving in the blood. In contrast, isogenic Pen^I strains were attenuated for such response and were out-competed by meningococci sensitive to penicillin G. Our results suggest that antibiotic resistance imposes a cost to the success of the pathogen and may potentially explain the lack of clonal expansion of Pen^I strains.

INTRODUCTION

Antibiotic resistance has become a major healthcare problem with the emergence of infectious agents that acquired multidrug resistance such as *Mycobacterium tuberculosis* or *Staphylococcus aureus*. In contrast, some important pathogens such as group A streptococci or *Neisseria meningitidis* have remained largely sensitive to the standard β-lactam antibiotic therapy despite several decades of antibiotic usage. *N. meningitidis* is exclusively an opportunistic human pathogen that may cause invasive infections (septicemia, pneumonia, and/or meningitis), but for which asymptomatic nasopharyngeal carriage is frequent (Cartwright et al., 1987).

Frequent DNA horizontal transfer occurs in the nasopharynx between different meningococcal isolates but also between *N. meningitidis* and other commensal *Neisseria* species (Maiden, 1993). Penicillin G remains the antibiotic of choice in the treatment of meningococcal infections (Quagliarello and Scheld, 1997). Plasmid-encoded β-lactamase has been rarely reported in *N. meningitidis* (Botha, 1988; Dillon et al., 1983). However, isolates with reduced susceptibility to penicillin G (Pen^I), usually defined by a minimal inhibitory concentration (MIC) for penicillin G ranging between 0.125 and 1 μg/ml, are becoming increasingly more frequent (Hughes et al., 1993) due to alterations in the *penA* gene encoding penicillin-binding protein (PBP) 2 through horizontal DNA transfer from commensal neisserial

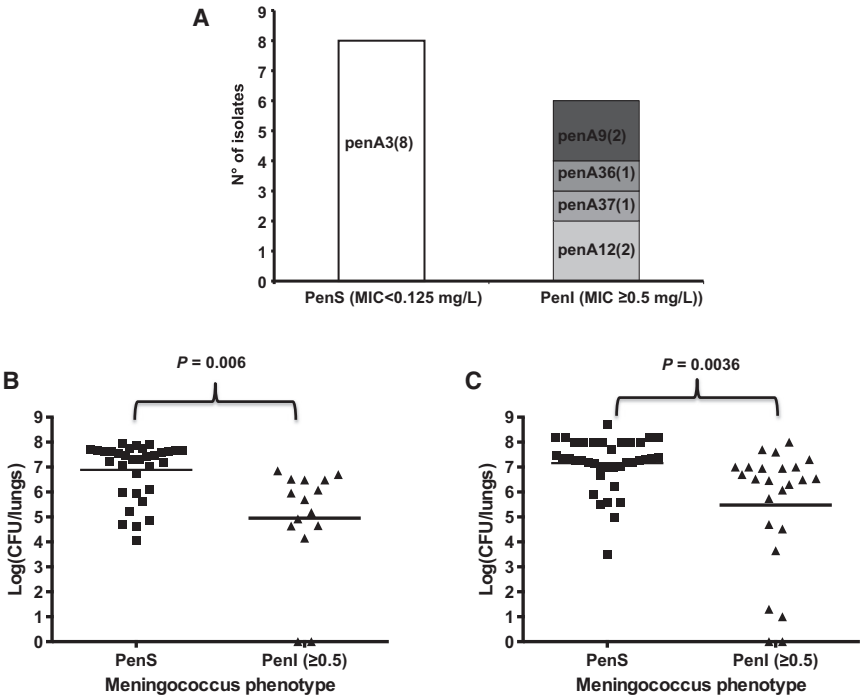

Figure 1. Comparative Virulence between Pen^S and Pen^I Meningococcal Clinical Isolates in an IAV-Infected BALB/c Mouse Model

(A) *penA* allele distrubution in the ST-11 clonal complex among Pen^S and Pen^I isolates. (B and C) Mice were intranasally challenged with standardized inocula of 5×10^7 cfu of eight and six different Pen^S and Pen^I meningococcal isolates, respectively, all belonging to the ST-11 clonal complex. Two Pen^I strains had a penicillin G MIC of 0.5 μg/ml, one of 0.75 μg/ml, and three of 1 μg/ml. At least four mice per strain were used. Results are presented as percentile boxes of bacterial cfu counts per strain in the lungs 3 (B) and 24 hr (C) after the bacterial infection and from three different experiments referred to the penicillin G MIC of each strain. Statistical analysis was done using the Mann-Whitney test. See also Figure S1.

species (Spratt et al., 1992). PBP2 contributes to the late stages of peptidoglycan (PG) biosynthesis, most likely in the transpeptidation reaction necessary for elongation of peptidoglycan (Spratt and Cromie, 1988). Critical alterations in the *penA* gene (encoding the PBP2) were directly linked to reduced susceptibility to penicillin G and were confirmed by transformation of altered *penA* genes into a susceptible strain (Antignac et al., 2003a). Sequencing of *penA* from a large collection of 1,670 meningococcal clinical isolates from 22 countries that spanned 60 years suggests that no clonal expansion of clinical isolates with reduced susceptibility to penicillin G was observed worldwide. Indeed, these clinical isolates were shown to belong to distinct genetic lineages and harbored different *penA* alleles. We have previously shown that alteration of PBP2 modifies the structure of meningococcal PG through the increase of proportions and amounts of pentapeptide-containing muropeptides (Antignac et al., 2003a). Bacterial signaling through PG may therefore be altered upon alterations of meningococcal PBP2 that confer the reduced susceptibility to penicillin G, affecting meningococcal acquisition and colonization at the portal of entry (the respiratory epithelium). The aim of this study was to analyze the biological consequences of PBP2 alteration among meningococcal strains with reduced susceptibility to penicillin G, which may explain the absence of expansion of these invasive isolates.

RESULTS

Alterations of PBP2 in Clinical Strains and Impact on the Experimental Infection in Mice

We first constructed a collection of 60 clinical isolates of *N. meningitidis* with reduced susceptibility to penicillin G in order to assess their virulence. Their MICs for penicillin G were determined by the Etest method (see Supplemental Information) and ranged between 0.125 μg/ml and 1 μg/ml. These isolates from invasive meningococcal infections were of different serogroups (40, 8, 11, and 1 isolates of the serogroups B, C, W135, and Y, respectively; Figure S1). Forty-one isolates belonged to the major clonal complexes encountered in invasive meningococcal infections (7, 6, 16, and 13 isolates of the clonal complexes ST-8, ST-11, ST-32, and ST-41/44, respectively, as determined by MSLT). Sequencing of the *penA* gene from some of these isolates showed diverse *penA* alleles (Figure 1A, data not shown, and Taha et al., 2007), indicating a highly heterogeneous structure of Pen^I isolates compared to the Pen^S strains (Figure 1A, data not shown, and Taha et al., 2007). Since Pen^I strains appear to result from independent *penA* acquisition/mosaic events in multiple different genetic lineages rather than expansion of one particular clone, we hypothesized that resistance might have a biological cost for meningococci (Dowson et al., 1989; Jabes et al., 1989). Isolates of the clonal complex ST-11 are genetically homogeneous and of high virulence (Zarantonelli et al., 2008). We therefore used the clinical isolates belonging to the ST-11 clonal complex showing Pen^S (eight strains) or Pen^I (six strains) phenotypes to challenge mice in the BALB/c mouse model of sequential IAV-meningococcal invasive respiratory challenge (Alonso et al., 2003). Bacterial counts in lungs were determined at 3 and 24 hr postinfection. Pen^S isolates yielded higher bacteria counts than Pen^I isolates (Figures 1B and 1C, $p = 0.006$ and $p = 0.0036$ at 3 and 24 hr postinfection, respectively). However, while these isolates belonged to the same clonal complex, they were not totally isogenic.

Impact of Eight Conserved Mutations in PBP2 Associated to Pen^I Phenotype on Peptidoglycan Composition

Therefore, to better study the impact of PBP2 alterations on meningococcal virulence, we constructed an isogenic Pen^I strain in the penicillin G susceptible strain LNP8013 by natural transformation of the altered *penA* allele carrying eight amino acid

Table 1. Muropeptide Composition of Parental Strain LNP8013 and Its Isogenic Pen^I Strain TR214/97

Muropeptide	Structure	LNP8013	TR214/97
1	Tri	1.47 ± 0.01	1.54 ± 0.02
2[b]	Tetra	12.07 ± 0.12	10.71 ± 0.11
4	Di	0.93 ± 0.01	1.06 ± 0.01
5[a]	Penta	1.50 ± 0.01	2.43 ± 0.02
6[b]	tetra OAc	5.76 ± 0.06	6.64 ± 0.07
7	Tetra-tri	1.63 ± 0.02	1.14 ± 0.01
8a	Tri (anh)	2.23 ± 0.02	2.00 ± 0.02
8b[b]	Tetra-tetra	10.18 ± 0.10	8.15 ± 0.08
9[a]	Tetra-penta	3.32 ± 0.03	7.09 ± 0.07
10	Tetra-tri OAc	0.95 ± 0.01	0.89 ± 0.01
12a[b]	Tetra-tetra OAc	15.14 ± 0.15	12.61 ± 0.13
12b[b]	Tetra (anh)	4.10 ± 0.04	2.74 ± 0.03
13[a]	Tetra-tetra-penta	2.15 ± 0.02	2.76 ± 0.03
14	Tetra-penta OAc	1.15 ± 0.01	1.31 ± 0.01
15	Tetra-tetra di-OAc	1.86 ± 0.02	1.66 ± 0.02
16[b]	Tetra-tetra-tetra OAc	7.20 ± 0.07	6.58 ± 0.07
18[a]	Tetra-penta di-OAc	4.80 ± 0.05	7.84 ± 0.08
19[b]	Tetra-tetra (anh)	5.15 ± 0.05	2.98 ± 0.03
20[b]	Tetra-tetra-tetra di-OAc	4.78 ± 0.05	4.11 ± 0.04
21	n.d.	4.41 ± 0.04	2.04 ± 0.02
22a	Tetra-tetra (anh) OAc	1.31 ± 0.01	2.21 ± 0.02
22b	Tetra-tetra-tetra tri-OAc	1.45 ± 0.01	2.28 ± 0.02
23	Tetra-tetra-tri (anh)	1.40 ± 0.01	1.74 ± 0.02
24	Tetra-tetra-tetra (anh) OAc	2.37 ± 0.02	2.44 ± 0.02
25	n.d.	1.25 ± 0.01	2.23 ± 0.02
26	Tetra-tetra-tetra (anh) di-OAc	1.44 ± 0.01	2.83 ± 0.03

Numbering of muropeptides correspond to the HPLC peaks presented in Figures S2 and S6. Structures were assigned after MALDI-TOF analysis of each muropeptide. Lines marked with a superscript "a" and "b" indicate muropeptides that increase and decrease, respectively, in percentage, in the Pen^I strain but also in the heterodiploid strains (Figure S6D) and the D,D-carboxypeptidase mutants AS7 and AS19 (Figure S6E).

substitutions common to Pen^I strains (Antignac et al., 2003a; Taha et al., 2007). Strain TR214/97 had a MIC of 0.5 μg/ml, clearly indicating that these eight amino acid substitutions were sufficient to confer a Pen^I phenotype to meningococci. No difference in growth rate in GCB medium was observed between Pen^I strain TR214/97 and its Pen^S parental strain LNP8013 (Figure S2A). Moreover, electron microscopy examination did not reveal any morphological changes (Figure S2A), indicating that there was no apparent biological cost for the Pen^I strain in vitro. However, the analysis of meningococcal Pen^S strain LNP8013 and isogenic Pen^I strain TR214/97 revealed modifications of peptidoglycan components in the isogenic Pen^I strain (Figure S2B and Table 1), particularly a similar increase in pentapeptide-containing muropeptides and a decrease in tetrapeptide-containing muropeptides including the tracheal cytotoxin (TCT) as previously described (Antignac et al., 2003a). Thus, clinical Pen^I isolates and the isogenic Pen^I strains shared common features (this work and Antignac et al., 2003a): a mosaic *penA* allele encoding a PBP2 leading to modifications of PG composition. Since PG fragments termed muropeptides such as TCT, the muramyl dipeptide (MDP), and the muramyl tripeptide (MTP) are pathogen-associated molecular patterns (PAMPs) sensed by pattern recognition receptors (PRRs) of the Nod-like receptor (NLR) family such as the cytosolic proteins Nod1 and Nod2 (Girardin et al., 2003a, 2003b; Magalhaes et al., 2005; Travassos et al., 2004), the response of the host to a pathogen will depend on the generation of such proinflammatory muropeptides.

Impaired In Vitro Inflammation of Isogenic Pen^I Strain

Thus, we next tested the Pen^S strain LNP8013 and its isogenic Pen^I variant TR214/97 in an epithelial infection model using the Hec1B cell line (Figure 2) or in a macrophage infection model (Figures S3A and S3B). While both strains adhered similarly to epithelial cells (data not shown), strain TR214/97 was impaired in the induction of IL-8 and TNF-α (Figures 2A and 2B). The distinct phenotype was specific for the epithelial model as macrophages responded similarly to both strains (Figures S3A and S3B). This is in agreement with the observation that epithelial cells downregulate Toll-like receptors (TLRs), relying mostly on NLRs to detect pathogens while macrophages use both types of PRRs. Accordingly, both strains produced identical LOS (data not shown), which is the major meningococcal PAMP sensed by macrophages through TLR4 (Zughaier et al., 2004). The phenotype on Hec1B cells could be recapitulated using highly purified PG (absence of LOS was tested by western blot; see Figure S3C) from both strains (Figures 2C and 2D). Experiments using siRNA to block Nod1 or TLR4 in Hec1B cells indicate that these cells responded to highly purified PG or MTP through Nod1 exclusively, excluding any contamination by other TLR ligands (Figure 3). These results also highlight that certain epithelial cells can sense PG through NLRs from the extracellular compartment without requiring prior internalization. These results also suggested that the PG modifications of the isogenic Pen^I strain TR214/97 resulted in the synthesis of a meningococcal PG with poor proinflammatory activity.

Pen^I Strain Is Impaired in IAV-Meningococcal Mouse Model

Next, we aimed at testing the isogenic pair on an in vivo model. *N. meningitidis* is a human-specific pathogen that does not normally infect mice. However, *N. meningitidis* becomes virulent and invasive in the sequential IAV-meningococcal invasive respiratory model (Alonso et al., 2003). Interestingly, this model mimics the human epidemiology where meningococcal disease outbreaks closely follow flu-like episodes (Rameix-Welti et al., 2009). In this IAV infection model, mice become susceptible to meningococcal disease at 7 days postinfection (Alonso et al., 2003). To better understand the effect of the IAV primary infection on the host, we performed microarray studies of the lungs of mice that were infected for 7 days with IAV and compared to naive mice (Figure S4). The analysis revealed that IAV infection led to suppression of the host immune system, in particular the downregulation of TLR4 (Figure S4B). Interestingly, Nod1 was not affected. We confirmed these observations independently by RT-PCR (Figure S4A). Thus, IAV primary infection

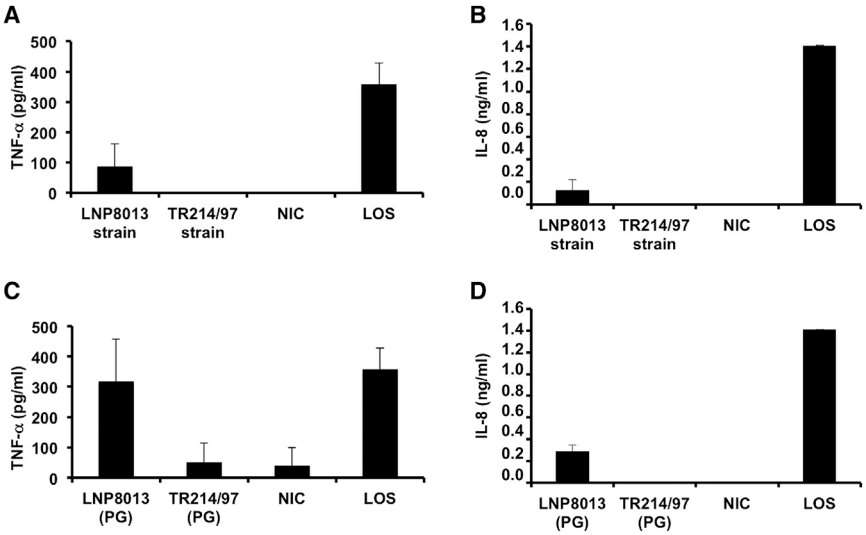

Figure 2. Cytokine Production by Hec1B Cells Stimulated by Purified PAMPs and Meningococcal Isogenic Pen^S and Pen^I Strains

(A–D) Hec1B cells were infected with isogenic strains LNP8013 (Pen^S) and TR214/97 (Pen^I), and productions of TNF-α (A) and IL-8 (B) were measured by ELISA. Similarly, Hec1B cells were stimulated with highly purified PG from strains LNP8013 and TR214/97, and TNF-α (C) and IL-8 (D) productions were measured. The isogenic TR214/97 Pen^I strain and its corresponding PG were impaired in inducing TNF-α and IL-8 productions compared to the Pen^S strain LNP8013 or its PG. Noninfected cells (NIC; only media) and cells stimulated with meningococcal LOS were used as negative and positive controls, respectively. Note that to have a positive response in Hec1B cells using, LOS, the equivalent of 8 × 10^7 bacteria of LOS was added while we had a response to 10 μg highly purified PG (equivalent to 10^7 bacteria, corresponding to 8-fold less). Data are presented as mean ± SEM. See also Figure S3.

predisposes the host to preferentially sense the Nod1 agonists compared to naive mice.

First, we performed a competition assay between the wild-type and its isogenic mutant (Figure S4C) and followed the colonization of the lungs at 3 hr and 24 hr. Despite a 9:1 ratio of the Pen^I over the Pen^S strain, after 24 hr the Pen^S had significantly displaced the Pen^I strain (around a 1:1 ratio). Next, we tested each strain individually in the same IAV model. The colonization of the lungs and bacteremia were scored also at 3 hr and 24 hr after meningococcal challenge. At time points beyond 24 hr, meningococci are spontaneously eliminated by lack of a suitable iron source in mice. Despite a reduced bacterial count in blood, suggesting impaired invasiveness and/or rapid clearance, these never reached statistical significance ($p = 0.163$ at 24 hr; Figure 4A). In contrast, bacterial loads in lungs were significantly lower for the strain TR214/97 when compared to wild-type strain LNP8013 ($p = 0.0378$; Figure 4A). These data suggested impaired capacity to colonize the respiratory tissues. Correlatively, significantly lower levels of TNF-α and IL-6 ($p = 0.02$ and $p = 0.01$, respectively) were detected in lungs of mice infected with strain TR214/97 at 3 hr after bacterial infection (Figure 4A). To check whether the TR214/97 strain phenotype was not due to secondary mutations, we restored the *penA* allele to a wild-type copy (strain R1). As illustrated in Figure S4D, the R1 strain recovered a wild-type virulence phenotype as it out-competed the mutant TR214/97 in the IAV infection model. The lower virulence of Pen^I isolates was further confirmed in another strain belonging to another meningococcal genetic lineage. We constructed an isogenic Pen^I strain (TR7lux) in the penicillin G susceptible strain LNP24198lux by natural transformation of the altered *penA* allele carrying eight amino acid substitutions common to Pen^I strains. After intranasal bacterial challenge, the bacterial colonization in lungs was estimated by measuring the photon emitted by bacteria on lungs, and dynamic imaging by Pen^I isogenic strains was significantly lower than that of the parent Pen^S strain ($p < 0.05$ in all tested time points; Figures S4E–S4G).

IAV-Meningococcal Disease Is Nod1 Dependent

To further characterize the role of PBP2 function in the virulence of meningococci, we tested whether the impaired virulence of Pen^I strain was related to PG sensing by the Nod proteins. As *N. meningitidis* is a Gram-negative bacteria carrying *meso*-DAP-type PG (thus a Nod1 agonist), we challenged C57BL/6J and C57BL/6J Nod1-deficient (*card4*$^{-/-}$), IAV-infected mice intranasally with strains LNP8013 and TR214/97 (Figures 4C and 4D). The Pen^I strain recapitulated an attenuated phenotype as in BALB/c mice (Figures 4A and 4C). However, no significant difference was observed in *card4*$^{-/-}$ mice in terms of cfu counts in lungs and blood, nor in the levels of the inflammatory cytokines in lungs (Figure 4D), indicating that the meningococcal PG plays a central role in virulence and establishing a local inflammatory response. The differences in bacterial load burden in the lungs, either in C57BL/6J or *card4*$^{-/-}$ mice, correlated with the macroscopic aspect of the lungs (Figure 5A). Analysis of bronchioalveolar lavage of lungs from C57BL/6J mice infected with either LNP8013 or TR214/97 revealed a significant difference in cell numbers (2.9 × 10^6 cells/ml versus 3.7 × 10^5 cells/ml, respectively; see Figure 5B). Morphological observation of the cell population recovered in the bronchioalveolar lavage (Figure 5B) showed an increase in polymorphic neutrophils (PMNs) in the mice infected with strain LNP8013 compared to the Pen^I strain TR214/97. To further characterize the cells infiltrating the lungs during infection, we sorted the bronchioalveolar lavages by flow cytometry. In C57BL/6J mice, the parental strain LNP8013 infection led to a significantly higher percentage of granulocytes compared to the isogenic Pen^I strain TR214/97 (Figure S5A). When gating these cells to further characterize them, we observed that the parental strain predominantly recruited cells positive for a 40 kDa antigen (Figure S5B) or positive for Ly-6G (Figure 5C) expressed by neutrophil cells that were absent from the lavage of mice challenged with TR214/97. Consistent with our infection experiments and the lung macroscopic observations, recruitment of cells positive for the 40 kDa antigen (Figures S5A and 5B) or positive for Ly-6G expressed by neutrophil cells was exclusively Nod1 dependent (Figure 5C).

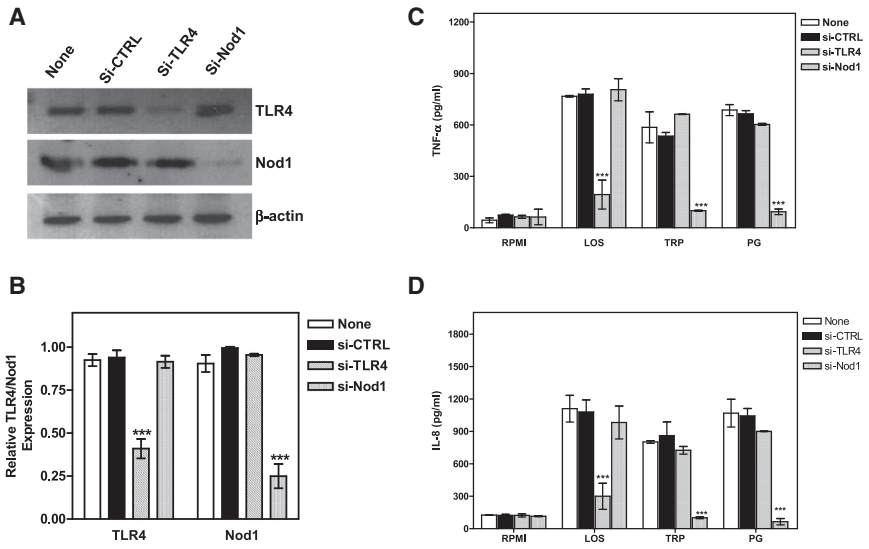

Figure 3. Small-Interference RNA Experiments in Hec1B Cells

(A–D) Cells were transfected with a control, a *tlr4*, or a *nod1* (also known as *card4*) siRNA. (A) RT-PCR expression analysis of Hec1B cells after 48 hr of siRNA treatment. β-actin was used an internal control. (B) Relative expression of *tlr4* and *nod1* using β-actin as a housekeeping gene. After 48 hr of treatment, TNF-α (C) and IL-8 (D) secretion after siRNA treatment of Hec1B cells, and stimulation with highly pure meningococcal LOS, HPLC purified N-acetylglucosamine-β(1,4)-N-acetyl-anydromuraminyl-L-alanyl-D-γ-glytamyl-*meso*-diaminopimelic acid (TRP) and highly pure meningococcal PG from the wild-type strain LNP8013. These experiments indicate that TRP and meningococcal PG were pure and mediated Hec1B response exclusively through Nod1. Furthermore, this response occurred by stimulation from the outside, indicating the Hec1B cells are able to internalize TRP and PG spontaneously. Statistical analysis was performed using Student's t test (two-tailed, unpaired; p values *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). Data are presented as mean ± SEM.

Finally, *N. meningitidis* is a human-specific pathogen. While human Nod1 preferentially detects MTP, mouse Nod1 better detects TCT, although the two Nod1 variants have some overlapping specificity (Magalhaes et al., 2005). Thus, we constructed transgenic human Nod1 mice (hNod1) in the background of the C57BL/6J *card4*$^{-/-}$ mice (Figures 6A and 6B). These mice expressed the transgenic gene in epithelia of different tissues (Figure 6A), the lung epithelia being the ones that expressed higher hNod1 gene. Furthermore, the hNod1 protein was functional, as these mice responded to MTP (Figure 6B) compared to non-transgenic liter mates, while both responded normally to MDP, the Nod2 ligand. Next, we compared the virulence of strains LNP8013 and TR214/97 using the IAV model in the transgenic mice. As illustrated in Figure 6C, strain TR214/97 colonized less well the lungs or the bloodstream. Similarly, the strain TR214/97 induced less damage of the lungs (Figure 5A) and fewer cytokines in the lungs (Figure 6C). We next explored whether primary epithelial cells from infected mice are able to produce proinflammatory cytokines in a Nod1-dependant manner. We performed bronchioalveolar washes in intranasally infected mice (wild-type and *card4*$^{-/-}$ mice) and sorted the bronchioalveolar washes by flow cytometry. In C57BL/6J mice, the parental strain LNP8013 infection led to a significantly higher percentage of epithelial cells producing TNF-α (percentage cells that are positive for both E-cadherin and TNF-α among cells that are positive for E-cadherin) compared to the isogenic Pen^{I} strain TR214/97 (Figures S5D–S5F).

Meningococcal Disease Relies on Peptidoglycan Modifications by D,D-Carboxypeptidases

To further understand the role of meningococcal PBP2 in PG metabolism, we constructed heterodiploid strains carrying a native *penA* allele and a dominant-negative *penA* allele (Figures S6A and S6B). The expression of the dominant-negative *penA* allele did not affect the production of native PBP2 (or the two other PBPs, PBP1 and PBP3; Figure S6C), nor did it affect its ability to bind penicillin G (Figure S6C). However, analysis of the PG composition by HPLC indicated that the dominant-negative *penA* allele also led to an increase of muropeptides carrying a pentapeptide and a generalized decrease of those carrying tetrapeptides (Figure S6D). Despite the fact that PBP2 is a class B high-molecular-weight PBP, our results with the isogenic TR214/97 and the dominant-negative *penA* allele suggested that the meningococcal PBP2 functioned in vivo as a D,D-carboxypeptidase.

However, several attempts to detect in vitro a D,D-carboxypeptidase activity using recombinant PBP2 have failed so far (data not shown). Alternatively, the decreased D,D-carboxypeptidase activity associated with altered PBP2 function could be an indirect effect. In *Escherichia coli*, the meningococcal PBP2 homolog PBP3, also known as FtsI, was shown to interact with the low-molecular-weight PBP7/PBP8, which are true D,D-carboxypeptidases (Romeis and Höltje, 1994). Meningococci have one low-molecular-weight PBP, PBP3, identifiable in fluorographic PBP assays (see Figure S6C). Additionally, two putative low-molecular-weight PBPs, PBP4 and PBP5, were revealed by sequence homology analysis of the complete genome. Hence, we tested whether the differences in PG composition between LNP8013 and TR214/97 were indirectly related to PBP3, PBP4, and PBP5. We constructed derivatives of LNP8013 and TR214/97, AS7 and AS19, respectively, in which the three genes *pbp3* (Nm665), *pbp4* (Nm962), and *pbp5* (Nm1095) were inactivated and analyzed the PG composition (Figure S6E). Interestingly, both derivatives, AS7 and AS19, produced identical PG composition characterized by a very high percentage of pentapeptide-carrying muropeptides (Figure S6E). These results suggested that the PBP2-related effects observed on the PG metabolism were probably indirect and mediated by the low-molecular-weight PBPs. Thus, we tested whether recombinant PBP2 and the major D,D-carboxypeptidase, PBP3, formed a stable protein complex. Indeed, PBP2 and PBP3 associated strongly with a kDa of 66 nM (Figure S6F). Finally, we tested whether both a LNP8013 heterodiploid strain carrying a dominant-negative *penA* allele and a LNP8013Δ*pbp3* mutant could

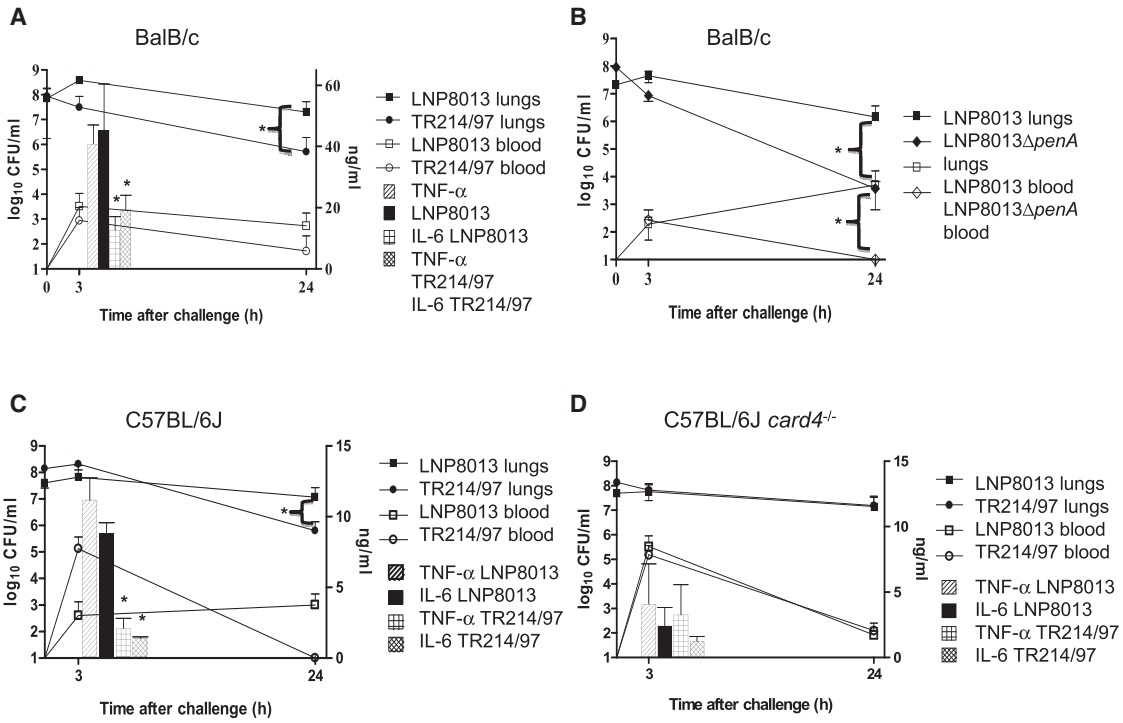

Figure 4. Comparative Virulence of *N. meningitidis* for IAV-Infected Mice

(A–D) BALB/c mice were infected with the wild-type Pen^S strain (LNP8013) and its isogenic Pen^I derivative (TR214/97) (A) or LNP8013Δ*penA*-22 (*penA*/*penA*::*erm* heteodiploid) (B). (C) and (D) are results from similar experiments conducted in IAV-infected C57BL/6J (WT) and Nod1$^{-/-}$ mice, respectively. Results are the means ± SEM (bars) of cfu counts in lungs and blood from three mice per time point and from two independent experiments. In the lungs, cfu counts per mice were obtained for both lungs homogenized in 1 ml of RPMI. Hence, cfu/ml is equivalent to cfu/lungs. TNF-α and IL-6 levels were measured in lungs after 3 hr of infection with strains LNP8013 or TR214/97. Cytokine levels are shown by histograms in (A), (C), and (D). Results are the means ± SEM (bars) of cytokine levels in lungs from three mice per time point and from two independent experiments. Statistical analysis was done using the Mann-Whitney test. "*" indicates $p \leq 0.05$. More details for each p value can be found in body of the text. See also Figures S4.

recapitulate the attenuated phenotype in the IAV model (Figures 4B and 6D, respectively). Indeed, both the heterodiploid strain and the PBP3 mutant were affected in colonizing lungs and persisted in the blood strain similarly to the Pen^I strain, suggesting that maintaining a balanced composition of its peptidoglycan is an important virulence trait of meningococcus.

DISCUSSION

The induction of the Nod1-dependent proinflammatory pathway relies on the ability of a bacterial pathogen (either invasive or extracellular) to translocate Gram-negative peptidoglycan to the intracellular environment (Girardin et al., 2003a). For example, *Helicobacter pylori*, an exclusively extracellular pathogen, uses a type four secretion system to deliver PG fragments into gastric epithelial cells (Viala et al., 2004). Alternatively, target cells can express transporters able to translocate through the membrane into the cytosol PG fragments. Several such transporters have been previously described, such as hPepT1, hPepT2, and SLC15A4 (Charrière et al., 2010; Lee et al., 2009; Vavricka et al., 2004). Furthermore, the human genome encodes for a homolog of the bacterial muropeptide transporter, AmpG (Park and Uehara, 2008). Although these transporters seem poorly expressed in epithelial cells, these can be induced under certain conditions such as endocytosis (Charrière et al., 2010; Vavricka et al., 2004). However, certain cell lines might present higher basal levels of these transporters, allowing for sensing PG fragments released by extracellular bacteria. Indeed, certain extracellular pathogens such as *Bordetella pertussis*, sharing the same ecological niche as *N. meningitidis*, are known to mediate inflammation though release of PG fragment while remaining extracellular. Finally, *N. meningitidis* releases large amounts of outer membrane vesicles that can serve as delivery system for PG. In fact, this mechanism has been shown to occur in *H. pylori* (Kaparakis et al., 2010).

The reduced virulence of Pen^I strains correlates with a decrease in tetrapeptide-containing muropeptides in meningococcal PG and a decrease in the proinflammatory response in the exposed host. The monomeric muropeptide anhydrous disaccharide-tetrapeptide, also known as the TCT, was first identified in gonococci and latter shown to reproduce the cytopathologic effect associated with whooping cough (Goldman and Herwaldt, 1985; Sinha and Rosenthal, 1980). TCT is known to have proinflammatory properties and to induce oxidative burst by phagocytic cells. Therefore, we reason that the Pen^I strains generate less TCT during host colonization. Since, in our mouse model, bacteremia is subsequent to a strong inflammatory response in the lungs (Alonso et al., 2003), the reduced virulence of the Pen^I strains could be explained by an impaired ability to generate enough TCT to allow transepithelial invasion to reach

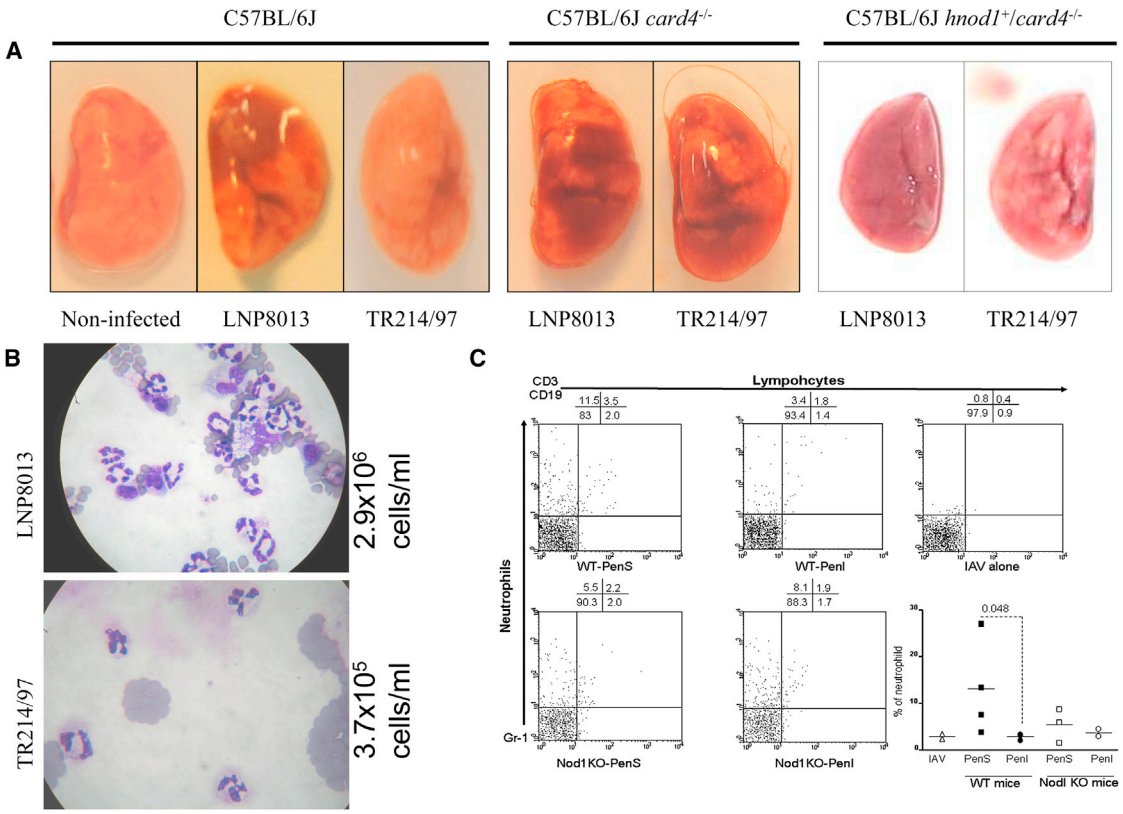

Figure 5. Recruitment of Cell Types and Morphological Aspects of Lungs from IAV-Infected Mice with an Isogenic Pair of Pen^S and Pen^I Strains

(A–C) C57BL/6J WT, Nod1$^{-/-}$, and hNod1 transgenic mice were challenged with Pen^S strain LNP8013 and its isogenic Pen^I derivative TR214/97. (A) Lungs were extracted and photographed 24 hr after bacterial infection. (B) Cells counts were determined in bronchioalveolar lavage from lungs of C57BL/6J mice infected with Pen^S strain LNP8013 and its isogenic Pen^I derivative TR214/97. The infection with the Pen^S strain resulted in 10-fold higher number of cells per ml in the lavage compared to its isogenic Pen^I derivative. Observation by optical microscopy of the cells present in the lavage indicated that strain LNP8013 induced essentially a recruitment of PMNs, which was drastically reduced for strain TR214/97. (C) Flow cytometry analysis of neutrophil in bronchioalveolar lavages (BAL). Neutrophil cells were scored as positive event for Gr-1 and also for a 40 kDa antigen expressed by neutrophil using a monoclonal antibody (clone 7/4) against this protein (Abcam) (see Supplemental Information). Controls were performed using unlabelled BAL or BAL labeled with secondary fluorescein goat anti-rat IgG alone. BALs were from C57BL/6J wild-type mice or Nod1 KO mice C57BL/6J *card4*$^{-/-}$. Mice were infected as in (A) by IAV alone or by IAV followed at day 7 by bacteria using Pen^S strain (LNP8013) or its isogenic Pen^I strain (TR214/97). Statistical analysis was done using unpaired t test or Mann-Whitney test ($p = 0.048$). See also Figures S5.

the blood and provoke meningococcemia. Indeed, TCT signaling has been recently shown to be dependent on Nod1 (Magalhaes et al., 2005). Our results showing that the Pen^I strain reduced virulence and altered cytokine production are both mouse and human Nod1 dependent are in accordance with this hypothesis. Furthermore, our results using the hNod1 transgenic mice argue against a mouse-specific phenotype, suggesting that Pen^I strains face the same constraints in humans. Our data strongly suggest that Nod1 signaling pathway is directly involved in meningococcal infection in our infection model and in humans. Accordingly, meningococci without lipooligosaccharide (LOS) still induce a vigorous inflammatory response in lungs of infected mice (Zarantonelli et al., 2006), reinforcing the notion that meningococcal PG has a central role in the pathogenesis of *N. meningitidis*. This small difference in the composition of PG between Pen^S and Pen^I isolates may therefore have important evolutionary consequences that contribute to more rapid invasiveness and transmission of Pen^S isolates, which might be a selective advantage that would explain the expanding of Pen^S but not that of Pen^I isolates. Accordingly, small but significant differences in the ability to induce early innate inflammatory response were recently reported between ancient and modern lineages of *Mycobacterium tuberculosis*. This difference was suggested to be associated with rapid disease progression and transmission, explaining the expansion of modern lineages in human populations (Portevin et al., 2011). The inflammation induced by *N. meningitidis* in the upper respiratory pathways could create the ideal environment for dissemination of *N. meningitidis* to a new host through extensive airway-prone droplets. Thus, in addition to the impaired virulence, Pen^I strains that are less proinflammatory would be also less fit for host-to-host dissemination.

Meningococcal disease, as well as other invasive bacterial infections, is favored by respiratory viral infections (at the portal of entry of these bacteria). In our mouse model, we show that IAV mediates immune suppression of the host, in particular

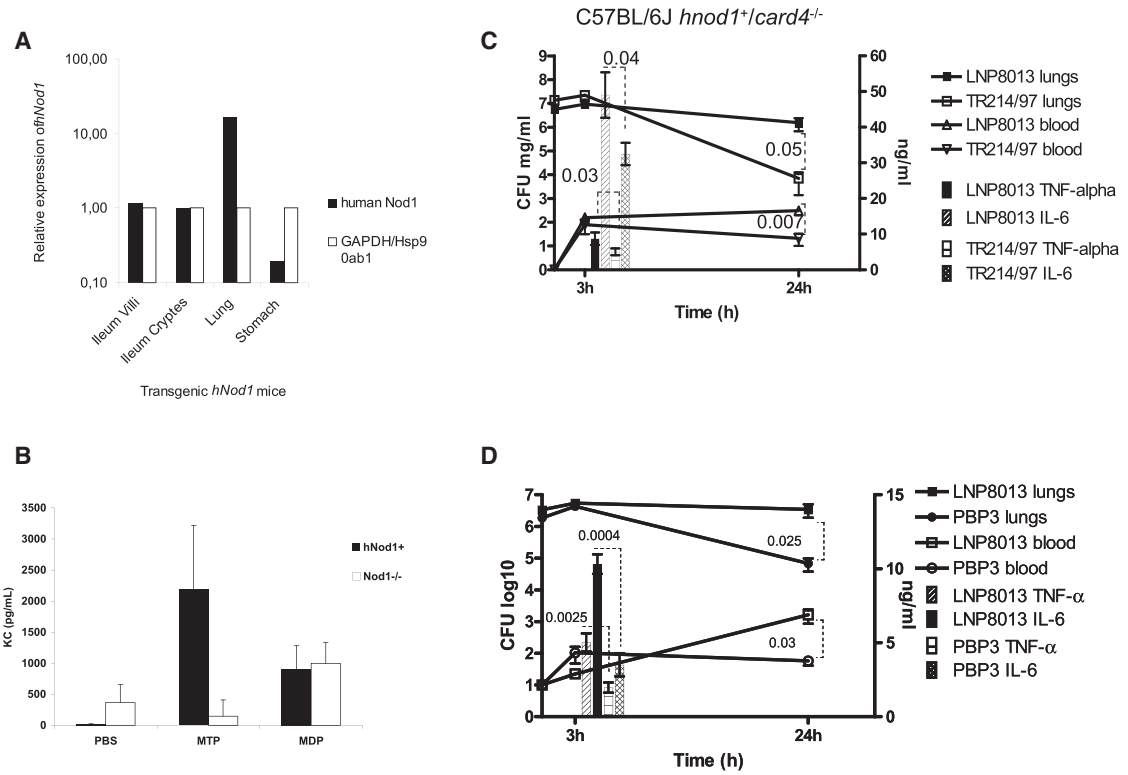

Figure 6. Human Nod1 Transgenic Mice and Meningococcal Virulence

(A) The expression of the transgene in hNod1-transgenic mice was assayed by LCM of the epithelial layer of ileum, lung, and stomach using as internal control the housekeeping genes *gapdh* and *hsp90*.

(B) The functionality of the transgenic mice was assayed by intraperitoneal injection of either PBS, the human Nod1 ligand muramyltripetide (MTP; 50 μg) and as control the Nod2 ligand, the muramyldipeptide (MDP; 50 μg). Two hours after intraperitoneal injection of the ligands, the level of KC was measured in the blood of mice. In the presence of the transgene, MTP induced a response that was absent from littermates devoid of both hNod1 and the mouse Nod1. In contrast, both transgenic and littermates responded normally to Nod2 stimulation.

(C) hNod1 transgenic mice were challenged by the IAV model with strains LNP8013 and TR214/97. Colony-forming units/ml were scored at 3 and 24 hr post-infection, while TNF-α and IL-6 levels were measured in lungs after 3 hr of infection. Statistical analysis was done using Student's t test (two-tailed, unpaired).

(D) Deletion of the meningococcal low-molecular-weight PBP3 in strain LNP8013 mimics the Pen^I phenotype. PBP3 was identified previously in STM screen as required for meningococci to sustain a bacteremia in the rat infant model (Sun et al., 2000). As shown in Figure 4, Pen^I strains are impaired in both lung colonization and bacteremia. We constructed an isogenic mutant of strain LNP8013 deleted for PBP3 and tested whether this mutant was also impaired for bacteremia using the IAV infection model. In the IAV infection model, the PBP3 mutant was impaired in colonizing the lungs, persisting in the blood, and in inducing an inflammatory response as measured by TNF-α and IL-6 secretion into the bronchioalveolar lavage. Data are presented as mean ± SEM. Statistical analysis was done using Student's t test (two-tailed, unpaired). See also Figure S6.

downregulation of the TLR4 pathway, unmasking a role for Nod1 in detecting meningococci. This is in agreement with recent reports showing viral suppression of TLRs through RIG-1 activation (Negishi et al., 2012). Also, it has been shown that viral infection leads to glucocorticoid production, also favoring immune suppression (Jamieson et al., 2010). Thus, our experimental model that mimics human epidemiology shows that meningococci exploit the host immune suppression to develop disease and reach new niches (lungs and blood). In the lungs, meningococci engage Nod1 in epithelial cells, leading to a Nod1-dependent meningococcal infection with recruitment of PMNs. This is consistent with a central role of Nod1 in recruiting PMNs (Dharancy et al., 2010; Masumoto et al., 2006). However, this response is unable to clear efficiently the Pen^S strains, probably due to the generalized immune suppression in respiratory pathways (Figure S4). Invasion of the bloodstream subsequently occurs with Pen^S strains. In agreement, viral infections have been shown to potentiate Nod1/2-mediated lethality of secondary bacterial infections (Kim et al., 2011). In contrast, Pen^I strains are less fit in inducing the initial Nod1 trigger in epithelial cells and recruitment of PMNs, resulting in a less productive persistence in the respiratory pathways and subsequent invasiveness.

Similar mechanisms of reduced susceptibility to penicillin G and other β-lactams have been described in other major human pathogens. For example, *Streptococcus pneumoniae* acquires mosaic genes encoding several PBPs to confer reduced susceptibility or even resistance to β-lactams. Recently, it has been shown that these variants display a fitness cost in an infant rat nasal colonization model (Trzcinski et al., 2006). Our results also provide an explanation for the lack of clonal expansion of meningococcal strains with reduced susceptibility to penicillin G. We propose the following model. *N. meningitidis* is a commensal of the nasopharynx flora that can take advantage of local inflammatory response in respiratory pathways to cross

the respiratory epithelium and to invade blood. Indeed, *N. meningitidis* invasiveness is tightly associated with an inflammatory response. Hence, penicillin G-reduced susceptibility carries a biological burden for the invasive strains.

Our results suggest that despite the increase in antibiotic resistance driven by their selective pressure, the fitness cost of resistance could be an ally in managing the problem of antibiotic resistance in bacteria.

EXPERIMENTAL PROCEDURES

Bacteria and Culture Conditions

Clinical isolates of *N. meningitidis* (n = 60) were from invasive meningococcal infections. MICs for penicillin G were determined by the Etest method (Solna, Sweden), and the serological typing was performed as previously described (Vázquez et al., 2003). Isolates were genotyped by multilocus sequence typing (MLST), as previously described (Maiden et al., 1998; Taha et al., 2004). Cultures were performed on GCB medium (Difco) containing Kellogg supplements (Kellogg et al., 1963) for 18 hr at 37°C under 5% CO_2. Transformation of *N. meningitidis* was done as previously described (Taha et al., 1996). *Escherichia coli* DH5 was used as a host for cloning experiments. *E. coli* cultures were done in Luria-Bertani medium supplemented with 40 μg/ml kanamycin and 300 μg/ml erythromycin when necessary. Detailed construction of all mutants is described in the Supplemental Information.

Peptidoglycan Purification

Bacterial strains were grown to exponential phase with or without penicillin G at different concentrations. Purification of peptidoglycan and structural analysis by high-pressure liquid chromatography (HPLC) were performed as described previously (Antignac et al., 2003b).

In Vitro Cellular Infection Assays

The Hec1B epithelial cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% inactivated fetal bovine serum. Hec1B cells (10^6 cells/well) were harvested and seeded into a 24-well plate with fresh medium without serum. The Hec1B monolayer was infected 24 hr later with 10^7/ml bacteria (LNP8013, Pen^S; TR214/97, isogenic Pen^I transformant), with human Nod1 ligand N-acetylglucosamine-β(1,4)-N-acetyl-anydromuraminyl-L-alanyl-D-γ-glytamyl-*meso*-diamino pimelic acid (TRP) purified by HPLC (10 nM) or with 10 μg highly purified PG (equivalent to 10^7 bacteria) from both strains (Antignac et al., 2003b). Noninfected cells (only medium) were used as a negative control. Highly purified meningococcal LOS (equivalent to 8×10^7 bacteria of strain LNP8013) was used as a positive control. After 4 hr of incubation, bacteria-free supernatants were used to measure the levels of TNF-α and IL-8 by the ELISA assays using anti-human TNF-α polyclonal antibody (Innogenetics) and anti-human IL-8 polyclonal antibody (PeproTech), respectively. The RAW264.7 macrophage cell line was grown as previously described (Boneca et al., 2007). Macrophages were infected with 10^7/ml bacteria (LNP8013 and TR214/97). After 1.5 hr and 8.5 hr of incubation, bacteria-free supernatants were used to measure the levels of TNF-α and IL-6 by the ELISA assays using Quantikine kits (R&D Systems). The Hec1B epithelial cell transfection with siRNA oligonucleotides is fully described in the Supplemental Information.

Mice Experiments

Mice were treated in accordance with French legislation and the Comité d'Hygiène, de Sécurité et des Conditions de Travail (Institut Pasteur, Paris, France). The virulence properties of meningococcal strains were tested in the mouse model of sequential influenza A virus (IAV)-bacterial infection (Alonso et al., 2003). This model was used in BALB/c (Charles River Laboratories), C57BL/6J, C57BL/6J *card4*$^{-/-}$ (Magalhaes et al., 2005; Viala et al., 2004), and transgenic C57BL/6J *hnod1*$^{+}$/*card4*$^{-/-}$ mice. Colony-forming unit counts were performed in samples of blood and lung homogenates (both lungs were taken in 1 ml of RPMI) from three mice at 3 and 24 hr after challenge. TNF-α and IL-6 levels were determined in lungs after 3 hr of bacterial challenge as previously described (Alonso et al., 2003) using Quantikine kits. The virulence of the PBP3 mutant was compared to the meningococcal wild-type isogenic strain LNP8013 in an IAV injection mouse model. Statistical analysis was performed using unpaired t test or Mann-Whitney test as appropriate. A p value of ≤ 0.05 was considered to be statistically significant. Dynamic imaging was performed in BALB/c (Société Janvier) IAV-infected mice as above. Intranasal bacteria challenge was performed and the bacterial infection images were acquired using an IVIS100 system (Xenogen Corp.) according to instructions from the manufacturer and as previously described (Szatanik et al., 2011).

Flow Cytometry

Bronchioalveolar lavage (BAL) fluid was recovered by introducing 1 ml of phosphate buffer using a 2.5 ml syringe. After several passages the liquid was withdrawn. Each BAL (100 μl) was incubated with anti-neutrophil antibody directed against a polymorphic 40 kDa antigen expressed by neutrophil cells (monoclonal antibody clone 7/4 Abcam), anti-mouse Ly-6G coupled to phycoerythrin (PE), and anti-CD3-FITC and anti-CD19-FITC (BD Biosciences) and analyzed using a FACSCalibur flow cytometer (BD Biosciences). To test the production of TNF-α in respiratory epithelial cells in BALs, flow cytometry was performed using monoclonal antibodies anti-murine E-cadherin-PE and anti-TNF-α-FITC. Prior to anti-TNF-α-FITC antibody labeling, cells from BALs were permeabilized for 15 min in saponin at a final concentration of 1%. Fluorescence was recorded from a total of 10,000 events per sample. The acquired fluorescence data were subsequently analyzed using WinMDI 2.9 software.

Full methods and associated references are available in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.04.016.

ACKNOWLEDGMENTS

Our research is supported by Institut Pasteur grants (PTR153 and PTR187), an ACI 0321 (Action Concertée Incitative Intégrée Microbiology) Grant from the Ministère chargé de la Recherche (INSERM No. MIC 0321), and a European Research Council starting grant (202283-PGNfromSHAPEtoVIR). A.S. was supported by a Marie Curie Intra-European Fellowship (No. 23188) within the Sixth European Community Framework Programme and Marie Curie European Reintegration Grants (No. 234876) within the Seventh European Community Framework Programme. M.E.G. was supported by an Institut Pasteur Roux fellowship. We would like to thank Jean-Michel Alonso, Pascale Cossart, and Marc Lecuit for discussion and critical reading of the manuscript. We would like to acknowledge the assistance of Isabelle Bonne and Marie-Christine Prévost for the transmission electron microscopy and Didier Blanot for providing lipid II.

Received: August 3, 2012
Revised: March 5, 2013
Accepted: April 30, 2013
Published: June 12, 2013

REFERENCES

Alonso, J.M., Guiyoule, A., Zarantonelli, M.L., Ramisse, F., Pires, R., Antignac, A., Deghmane, A.E., Huerre, M., van der Werf, S., and Taha, M.K. (2003). A model of meningococcal bacteremia after respiratory superinfection in influenza A virus-infected mice. FEMS Microbiol. Lett. *222*, 99–106.

Antignac, A., Boneca, I.G., Rousselle, J.C., Namane, A., Carlier, J.P., Vázquez, J.A., Fox, A., Alonso, J.M., and Taha, M.K. (2003a). Correlation between alterations of the penicillin-binding protein 2 and modifications of the peptidoglycan structure in *Neisseria meningitidis* with reduced susceptibility to penicillin G. J. Biol. Chem. *278*, 31529–31535.

Antignac, A., Rousselle, J.C., Namane, A., Labigne, A., Taha, M.K., and Boneca, I.G. (2003b). Detailed structural analysis of the peptidoglycan of the human pathogen *Neisseria meningitidis*. J. Biol. Chem. *278*, 31521–31528.

Boneca, I.G., Dussurget, O., Cabanes, D., Nahori, M.A., Sousa, S., Lecuit, M., Psylinakis, E., Bouriotis, V., Hugot, J.P., Giovannini, M., et al. (2007). A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. Proc. Natl. Acad. Sci. USA *104*, 997–1002.

Botha, P. (1988). Penicillin-resistant *Neisseria meningitidis* in southern Africa. Lancet *1*, 54.

Cartwright, K.A., Stuart, J.M., Jones, D.M., and Noah, N.D. (1987). The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. Epidemiol. Infect. *99*, 591–601.

Charrière, G.M., Ip, W.E., Dejardin, S., Boyer, L., Sokolovska, A., Cappillino, M.P., Cherayil, B.J., Podolsky, D.K., Kobayashi, K.S., Silverman, N., et al. (2010). Identification of Drosophila Yin and PEPT2 as evolutionarily conserved phagosome-associated muramyl dipeptide transporters. J. Biol. Chem. *285*, 20147–20154.

Dharancy, S., Body-Malapel, M., Louvet, A., Berrebi, D., Gantier, E., Gosset, P., Viala, J., Hollebecque, A., Moreno, C., Philpott, D.J., et al. (2010). Neutrophil migration during liver injury is under nucleotide-binding oligomerization domain 1 control. Gastroenterology *138*, 1546–1556.

Dillon, J.R., Pauzé, M., and Yeung, K.H. (1983). Spread of penicillinase-producing and transfer plasmids from the gonococcus to *Neisseria meningitidis*. Lancet *1*, 779–781.

Dowson, C.G., Jephcott, A.E., Gough, K.R., and Spratt, B.G. (1989). Penicillin-binding protein 2 genes of non-beta-lactamase-producing, penicillin-resistant strains of *Neisseria gonorrhoeae*. Mol. Microbiol. *3*, 35–41.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jéhanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zähringer, U., et al. (2003a). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science *300*, 1584–1587.

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

Goldman, W.E., and Herwaldt, L.A. (1985). *Bordetella pertussis* tracheal cytotoxin. Dev. Biol. Stand. *61*, 103–111.

Hughes, J.H., Biedenbach, D.J., Erwin, M.E., and Jones, R.N. (1993). E test as susceptibility test and epidemiologic tool for evaluation of *Neisseria meningitidis* isolates. J. Clin. Microbiol. *31*, 3255–3259.

Jabes, D., Nachman, S., and Tomasz, A. (1989). Penicillin-binding protein families: evidence for the clonal nature of penicillin resistance in clinical isolates of pneumococci. J. Infect. Dis. *159*, 16–25.

Jamieson, A.M., Yu, S., Annicelli, C.H., and Medzhitov, R. (2010). Influenza virus-induced glucocorticoids compromise innate host defense against a secondary bacterial infection. Cell Host Microbe *7*, 103–114.

Kaparakis, M., Turnbull, L., Carneiro, L., Firth, S., Coleman, H.A., Parkington, H.C., Le Bourhis, L., Karrar, A., Viala, J., Mak, J., et al. (2010). Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. Cell. Microbiol. *12*, 372–385.

Kellogg, D.S., Jr., Peacock, W.L., Jr., Deacon, W.E., Brown, L., and Pirkle, D.I. (1963). *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. *85*, 1274–1279.

Kim, Y.G., Park, J.H., Reimer, T., Baker, D.P., Kawai, T., Kumar, H., Akira, S., Wobus, C., and Núñez, G. (2011). Viral infection augments Nod1/2 signaling to potentiate lethality associated with secondary bacterial infections. Cell Host Microbe *9*, 496–507.

Lee, J., Tattoli, I., Wojtal, K.A., Vavricka, S.R., Philpott, D.J., and Girardin, S.E. (2009). pH-dependent internalization of muramyl peptides from early endosomes enables Nod1 and Nod2 signaling. J. Biol. Chem. *284*, 23818–23829.

Magalhaes, J.G., Philpott, D.J., Nahori, M.A., Jéhanno, M., Fritz, J., Le Bourhis, L., Viala, J., Hugot, J.P., Giovannini, M., Bertin, J., et al. (2005). Murine Nod1 but not its human orthologue mediates innate immune detection of tracheal cytotoxin. EMBO Rep. *6*, 1201–1207.

Maiden, M.C. (1993). Population genetics of a transformable bacterium: the influence of horizontal genetic exchange on the biology of *Neisseria meningitidis*. FEMS Microbiol. Lett. *112*, 243–250.

Maiden, M.C., Bygraves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., et al. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA *95*, 3140–3145.

Masumoto, J., Yang, K., Varambally, S., Hasegawa, M., Tomlins, S.A., Qiu, S., Fujimoto, Y., Kawasaki, A., Foster, S.J., Horie, Y., et al. (2006). Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment *in vivo*. J. Exp. Med. *203*, 203–213.

Negishi, H., Yanai, H., Nakajima, A., Koshiba, R., Atarashi, K., Matsuda, A., Matsuki, K., Miki, S., Doi, T., Aderem, A., et al. (2012). Cross-interference of RLR and TLR signaling pathways modulates antibacterial T cell responses. Nat. Immunol. *13*, 659–666.

Park, J.T., and Uehara, T. (2008). How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). Microbiol. Mol. Biol. Rev. *72*, 211–227.

Portevin, D., Gagneux, S., Comas, I., and Young, D. (2011). Human macrophage responses to clinical isolates from the *Mycobacterium tuberculosis* complex discriminate between ancient and modern lineages. PLoS Pathog. *7*, e1001307.

Quagliarello, V.J., and Scheld, W.M. (1997). Treatment of bacterial meningitis. N. Engl. J. Med. *336*, 708–716.

Rameix-Welti, M.A., Zarantonelli, M.L., Giorgini, D., Ruckly, C., Marasescu, M., van der Werf, S., Alonso, J.M., Naffakh, N., and Taha, M.K. (2009). Influenza A virus neuraminidase enhances meningococcal adhesion to epithelial cells through interaction with sialic acid-containing meningococcal capsules. Infect. Immun. *77*, 3588–3595.

Romeis, T., and Höltje, J.V. (1994). Specific interaction of penicillin-binding proteins 3 and 7/8 with soluble lytic transglycosylase in *Escherichia coli*. J. Biol. Chem. *269*, 21603–21607.

Sinha, R.K., and Rosenthal, R.S. (1980). Release of soluble peptidoglycan from growing conococci: demonstration of anhydro-muramyl-containing fragments. Infect. Immun. *29*, 914–925.

Spratt, B.G., and Cromie, K.D. (1988). Penicillin-binding proteins of gram-negative bacteria. Rev. Infect. Dis. *10*, 699–711.

Spratt, B.G., Bowler, L.D., Zhang, Q.Y., Zhou, J., and Smith, J.M. (1992). Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. J. Mol. Evol. *34*, 115–125.

Sun, Y.H., Bakshi, S., Chalmers, R., and Tang, C.M. (2000). Functional genomics of *Neisseria meningitidis* pathogenesis. Nat. Med. *6*, 1269–1273.

Szatanik, M., Hong, E., Ruckly, C., Ledroit, M., Giorgini, D., Jopek, K., Nicola, M.A., Deghmane, A.E., and Taha, M.K. (2011). Experimental meningococcal sepsis in congenic transgenic mice expressing human transferrin. PLoS ONE *6*, e22210.

Taha, M.K., Giorgini, D., and Nassif, X. (1996). The *pilA* regulatory gene modulates the pilus-mediated adhesion of *Neisseria meningitidis* by controlling the transcription of pilC1. Mol. Microbiol. *19*, 1073–1084.

Taha, M.K., Giorgini, D., Ducos-Galand, M., and Alonso, J.M. (2004). Continuing diversification of *Neisseria meningitidis* W135 as a primary cause of meningococcal disease after emergence of the serogroup in 2000. J. Clin. Microbiol. *42*, 4158–4163.

Taha, M.K., Vázquez, J.A., Hong, E., Bennett, D.E., Bertrand, S., Bukovski, S., Cafferkey, M.T., Carion, F., Christensen, J.J., Diggle, M., et al. (2007). Target gene sequencing to characterize the penicillin G susceptibility of *Neisseria meningitidis*. Antimicrob. Agents Chemother. *51*, 2784–2792.

Travassos, L.H., Girardin, S.E., Philpott, D.J., Blanot, D., Nahori, M.A., Werts, C., and Boneca, I.G. (2004). Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep. *5*, 1000–1006.

Trzcinski, K., Thompson, C.M., Gilbey, A.M., Dowson, C.G., and Lipsitch, M. (2006). Incremental increase in fitness cost with increased beta -lactam

resistance in pneumococci evaluated by competition in an infant rat nasal colonization model. J. Infect. Dis. *193*, 1296–1303.

Vavricka, S.R., Musch, M.W., Chang, J.E., Nakagawa, Y., Phanvijhitsiri, K., Waypa, T.S., Merlin, D., Schneewind, O., and Chang, E.B. (2004). hPepT1 transports muramyl dipeptide, activating NF-kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. Gastroenterology *127*, 1401–1409.

Vázquez, J.A., Arreaza, L., Block, C., Ehrhard, I., Gray, S.J., Heuberger, S., Hoffmann, S., Kriz, P., Nicolas, P., Olcen, P., et al. (2003). Interlaboratory comparison of agar dilution and Etest methods for determining the MICs of antibiotics used in management of Neisseria meningitidis infections. Antimicrob. Agents Chemother. *47*, 3430–3434.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Mémet, S., Huerre, M.R., Coyle, A.J., et al. (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. Nat. Immunol. *5*, 1166–1174.

Zarantonelli, M.L., Huerre, M., Taha, M.K., and Alonso, J.M. (2006). Differential role of lipooligosaccharide of *Neisseria meningitidis* in virulence and inflammatory response during respiratory infection in mice. Infect. Immun. *74*, 5506–5512.

Zarantonelli, M.L., Lancellotti, M., Deghmane, A.E., Giorgini, D., Hong, E., Ruckly, C., Alonso, J.M., and Taha, M.K. (2008). Hyperinvasive genotypes of *Neisseria meningitidis* in France. Clin. Microbiol. Infect. *14*, 467–472.

Zughaier, S.M., Tzeng, Y.L., Zimmer, S.M., Datta, A., Carlson, R.W., and Stephens, D.S. (2004). *Neisseria meningitidis* lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway. Infect. Immun. *72*, 371–380.