

The TLR2-MyD88-NOD2-RIPK2 signalling axis regulates a balanced pro-inflammatory and IL-10-mediated anti-inflammatory cytokine response to Gram-positive cell walls

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Summary

Systemic infection with *Streptococcus pneumoniae* is associated with a vigorous pro-inflammatory response to structurally complex cell wall fragments (PnCw) that are shed during cell growth and antibiotic-induced autolysis. Consistent with previous studies, inflammatory cytokine production induced by PnCw was dependent on TLR2 but independent of NOD2, a cytoplasmic NLR protein. However, in parallel with the pro-inflammatory response, we found that PnCw also induced prodigious secretion of anti-inflammatory IL-10 from macrophages. This response was dependent on TLR2, but also involved NOD2 as absence of NOD2-reduced IL-10 secretion in response to cell wall and translated into diminished downstream effects on IL-10-regulated target gene expression. PnCw-mediated production of IL-10 via TLR2 required RIPK2 a kinase required for NOD2 function, and MyD88 but differed from that known for zymosan in that ERK pathway activation was not detected. As mutations in

NOD2 are linked to aberrant immune responses, the temporal and quantitative effects of activation of the TLR2-NOD2-RIPK2 pathway on IL-10 secretion may affect the balance between pro- and anti-inflammatory responses to Gram-positive bacteria.

Introduction

IL-10 secretion from T cells, macrophages and dendritic cells is an essential regulatory mechanism to temper excessive inflammatory responses (Murray, 2006). Mice lacking IL-10 are extremely sensitive to a wide variety of pro-inflammatory stimuli including LPS, and systemic infections with bacteria and parasites that provoke a strong inflammatory response (Murray, 2006). In all cases, IL-10 is required to restrain the inflammatory response and protect the host against the damaging effects of pro-inflammatory cytokines. Systemic infection with *S. pneumoniae* provokes a generalized inflammatory response linked with devastating neurological sequelae and a fatality rate of ~15% (Tuomanen *et al.*, 1995). Administration of purified pneumococcal cell wall (PnCw) recapitulates the majority of the inflammatory responses observed in systemic *S. pneumoniae* infection, suggesting that the pro-inflammatory properties of PnCw are a primary driver of inflammatory pathology (Moreillon and Majcherczyk, 2003; Fillon *et al.*, 2006; Orihuela *et al.*, 2006). Because PnCw is mainly composed of peptidoglycan, teichoic acid, cell wall linked proteins and lipoteichoic acids, TLR2 is considered especially important in the process of PnCw detection by macrophages and dendritic cells that are then stimulated to produce inflammatory cytokines (Yoshimura *et al.*, 1999). IL-10 can modulate PnCw induced inflammation (Orihuela *et al.*, 2006), but the mechanism leading to IL-10 production is entirely unclear.

Modulation of inflammation has been suggested to involve NOD2, a cytoplasmic protein of the larger NLR family (Inohara and Nunez, 2003; Inohara *et al.*, 2005; Murray, 2005; Strober *et al.*, 2006; Kanneganti *et al.*, 2007). For example, single nucleotide polymorphisms in *NOD2* have been observed at an increased frequency in Crohn's disease (Lesage *et al.*, 2002). However, several

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loss-of-function mice in the *Nod2* gene do not have gut immunopathology when maintained under normal conditions (Pauleau and Murray, 2003; Kobayashi *et al.*, 2005; Maeda *et al.*, 2005; Mariathasan *et al.*, 2006; Barreau *et al.*, 2007). These data are consistent with the fact that some people have mutations in one or both *NOD2* alleles but have no clinical inflammatory bowel disease (Hugot *et al.*, 2007). Thus *NOD2* likely plays a role in more complex pathways modulating pro-inflammatory responses (Strober *et al.*, 2007; Xavier and Podolsky, 2007).

NOD2-deficient mice, their immune cells and human cells bearing predicted loss-of-function *NOD2* alleles, are non-responsive to muramyl dipeptide (MDP), a 'minimal' component of bacterial peptidoglycan able to induce pro-inflammatory responses. A second pathway of MDP sensing appears to be involved in IL-1 β processing and is mediated by NLR family members Cryopyrin (NLRP3) and Nalp1 (NLRP1) (Faustin *et al.*, 2007; Pan *et al.*, 2007; Marina-Garcia *et al.*, 2008). At this stage it is unclear whether human cells bearing two copies of the *NOD2* frame-shift mutation are completely non-responsive to MDP because the truncated mutant protein should still be expressed. Compared with other bacterial components such as LPS, peptidoglycan or CpG DNA, MDP is a very weak agonist of myeloid-derived cells, especially when considered by molar comparison. However, MDP synergizes with other TLR agonists to stimulate cytokine, chemokine and nitric oxide production. This effect is the basis of variants of the 'MDP synergy' assay, a common 'readout' of *NOD2* function. In contrast to the lack of MDP responsiveness, macrophages from all *NOD2*-deficient mice have been generally reported to have 'normal' responses to highly defined TLR agonists. At present, the specific roles of MDP, the origin of MDP in mammalian infection systems and the link between *NOD2* and MDP remain unresolved.

In the studies described here, we observed that TLR2 and *NOD2* were together responsible for IL-10 production when macrophages were exposed to PnCw. By contrast, inflammatory cytokine production in response to PnCw was TLR2 dependent and *NOD2*-independent. Our studies reveal an unexpected pathway of signal integration in response to a complex microbial product that stimulates multiple signalling pathways in macrophages.

Results

When *S. pneumoniae* grow *in vivo*, cell wall components are released into the host environment by several processes: cell wall turnover that occurs during normal growth, antibiotics that induce autolysis, and subpopulations that undergo spontaneous autolysis as a mechanism of interbacterial gene transfer. The latter process

lead in part to the discovery of DNA as the source of genetic information. Released cell wall fragments stimulate a complex pathway of local and systemic inflammatory responses including neuronal damage and memory loss (Tuomanen *et al.*, 1995). Because cell wall fragments derived from yeast (zymosan) induce robust IL-10 secretion from myeloid-derived cells (Dillon *et al.*, 2004; 2006; Rogers *et al.*, 2005; Slack *et al.*, 2007), we tested if purified PnCw (Fillon *et al.*, 2006) also induced IL-10 production from bone marrow-derived macrophages (BMDMs). PnCw induced previously unsuspected high amounts of IL-10 (Fig. 1). As purified PnCw is composed primarily of peptidoglycan, a TLR2 agonist, this response was predictably TLR2 dependent (Fig. 1). In parallel experiments we also observed that IL-10 and IL-10 mRNA production in response to PnCw was also dependent on *NOD2* (Figs 1B, D and E). The reduction in IL-10 production from PnCw-stimulated *NOD2*-deficient macrophages was not to the same extent as *Tlr2*^{-/-} BMDMs but nevertheless highly reproducible. We also observed that BMDMs derived from *Tlr2*^{-/-};*Nod2*^{-/-} mice secreted undetectable IL-10, demonstrating that TLR2 and *NOD2* might function in the same or related pathways for IL-10 production (Figs 1B, D and E). By contrast, IL-10 production in response to LPS was indistinguishable in *Tlr2*^{-/-}, *Nod2*^{-/-} or *Tlr2*^{-/-};*Nod2*^{-/-} cells (Fig. 1C). We tested if *NOD2* was required for IL-10 production in response to Pam₃CSK4, a synthetic lipid that signals via exclusively TLR2. We observed that *Nod2*^{-/-} BMDMs produced IL-10 in response to Pam₃CSK4 similar to control cells (data not shown). In contrast, Pam₃CSK4 did not induce IL-10 in *Tlr2*^{-/-} or *Tlr2*^{-/-};*Nod2*^{-/-} cells as expected (data not shown). *NOD2* was therefore not required for the 'canonical' TLR2 activation pathway stimulated by Pam₃CSK4 but was important to the physiological PnCw stimulus.

Because PnCw is a complex macromolecule, we tested its cellular fate after exposure to BMDMs. We visualized PnCw fragments in macrophages through the use of FITC-labelled PnCw (Fig. 1F and G). Macrophages consumed all the PnCw over a period of ~24 h and thereafter, the PnCw remained internalized and apparently unchanged over 7 days when the experiments were terminated. Comparing these data with the kinetics of cytokine secretion, we suspect that macrophages are stimulated with early, external contact with PnCw. By contrast, phagocytosed PnCw appeared to persist within macrophages. Further experiments will be necessary to determine if macrophages can generate MDP from the PnCw and any signalling connections between *NOD2* and in-dwelling PnCw.

We next asked how broadly *NOD2* influenced overall macrophage transcriptional responses to PnCw. We performed transcriptome analysis of BMDMs derived from background-matched C57BL/6 *Nod2*^{+/+} or *Nod2*^{-/-} mice

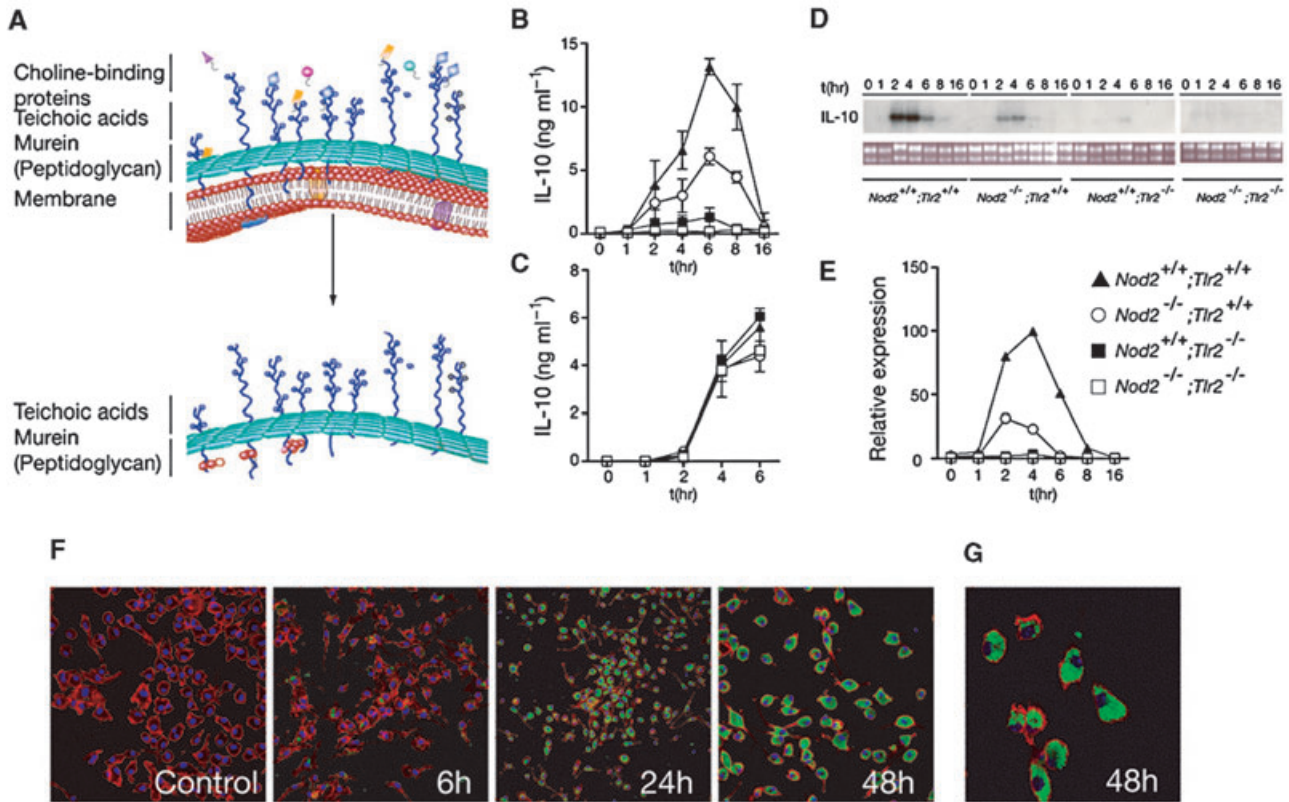


Fig. 1. Combined signals from TLR2 and NOD2 are required for PnCw-induced IL-10 production.

A. Schematic representation of the composition of PnCw obtained by biochemical purification from whole lysed pneumococci. The upper part of the figure is cartoon of the bacteria cell membrane and wall indicating that the wall composed primarily of peptidoglycan, to which multiple proteins, lipopeptides and lipids are tethered, surrounds the cell membrane of pneumococci. Following purification, PnCw is predominantly peptidoglycan and teichoic acids. This fraction is particulate in nature, analogous to zymosan fractions from yeast cell walls.

B. IL-10 production from BMDMs stimulated with PnCw measured over time by ELISA. Results are combined from independent experiments ($n = 7$).

C. IL-10 production from LPS stimulation of BMDMs from the same genotypes used in (B).

D. Northern blotting analysis of IL-10 mRNA over time (h) following PnCw stimulation of BMDMs from the indicated genotypes. Data from *Nod2*^{-/-}; *Tlr2*^{-/-} BMDMs were obtained from a different part of the gel as shown the separation from the other part of the gel.

E. qRT-PCR analysis of IL-10 mRNA production following PnCw stimulation. Data points are averages of duplicate samples. Data are representative of three independent experiments.

F. Confocal microscopy to follow the fate of PnCw after contact with BMDMs. FITC-labelled PnCw was added to C57BL/6 BMDMs and followed over time. Representative images at times 0, 5, 24 and 48 h are shown. At each time, cultures were fixed and labelled with phalloidin to visualize actin. Note that the FITC signal is not visible until sufficient PnCw particle have been phagocytosed and concentrated inside macrophages (24 h). All images were collected using a 10 \times objective, except the 48 h time point (20 \times).

G. Higher power image of PnCw inside BMDMs at 48 h (40 \times objective).

stimulated for 0, 1 or 4 h with PnCw at a concentration and conditions identical to that used for the experiments in Fig. 1. We analysed the data first by principal component analysis (data not shown) and then by ordering gene expression according to degree of induction relative to the untreated (0 h) time point data (Table 1). We observed that the absence of NOD2 had limited effects on the overall inflammatory response to PnCw (Table 1, Fig. 2A) consistent with the notion that other receptors, especially TLR2, mediate a strong cellular activation by PnCw. Of the mRNAs whose expression was altered, we noted that IL-10 mRNA expression was reduced in NOD2-deficient cells relative to controls consistent with the ELISA and mRNA data shown in Fig. 1. Because IL-10 has essential

autocrine-paracrine inhibitory effects on activated macrophages, we next asked if a panel of known downstream target genes of IL-10 signalling (Lang *et al.*, 2002; El Kasmi *et al.*, 2007) were affected by the reduction of IL-10 after PnCw stimulation of NOD2-deficient macrophages. We observed that expression of multiple IL-10 target genes was lowered relative to controls in NOD2-deficient macrophages at 4 h post stimulation (Fig. 2B). This is consistent with the data that PnCw stimulates IL-10 production in a NOD2-dependent way that leads, in part, to autocrine-paracrine downstream effects on IL-10-responsive gene expression.

By contrast to the requirement for both TLR2 and NOD2 in IL-10 production, we observed that all tested

Table 1. Gene expression increased by PnCW treatment.

| Gene | Rank (WT) | Fold (4 h) | Rank (<i>Nod2</i> ^{-/-}) | Fold (4 h) | <i>P</i> (time) | <i>P</i> (genotype) |
|------------------|-----------|------------|-------------------------------------|------------|-----------------|---------------------|
| <i>Gm 1960</i> | 1 | 222.14 | 1 | 205.56 | 1.41E-13 | 0.5314 |
| <i>Irg1</i> | 2 | 124.62 | 2 | 145.62 | 4.32E-11 | 0.8515 |
| <i>Cxcl1</i> | 3 | 102.85 | 4 | 111.82 | 2.17E-11 | 0.3880 |
| <i>Tnf</i> | 4 | 82.04 | 3 | 124.60 | 1.02E-11 | 0.8967 |
| <i>Cxcl1</i> | 5 | 78.06 | 5 | 102.79 | 4.75E-11 | 0.3036 |
| <i>Ccl3</i> | 6 | 77.61 | 6 | 76.38 | 4.39E-11 | 0.9913 |
| <i>Saa3</i> | 7 | 76.27 | 8 | 54.01 | 2.37E-14 | 0.1997 |
| <i>Ptgs2</i> | 8 | 57.72 | 16 | 29.27 | 4.13E-09 | 0.9574 |
| <i>Ccl4</i> | 9 | 54.47 | 14 | 32.23 | 3.61E-12 | 0.6201 |
| <i>Il1b</i> | 10 | 51.93 | 15 | 32.00 | 8.30E-07 | 0.7485 |
| <i>Gpr109a</i> | 11 | 51.73 | 10 | 37.09 | 7.80E-14 | 0.2307 |
| <i>Slc7a11</i> | 12 | 51.72 | 19 | 24.34 | 4.11E-14 | 0.6030 |
| <i>Ptgs2</i> | 13 | 51.63 | 46 | 16.03 | 8.02E-08 | 0.1154 |
| <i>Il1a</i> | 14 | 45.04 | 50 | 15.37 | 6.39E-07 | 0.7571 |
| <i>Il6</i> | 15 | 44.03 | 11 | 35.24 | 1.02E-06 | 0.3773 |
| <i>Socs3</i> | 16 | 43.81 | 13 | 32.65 | 2.46E-11 | 0.2271 |
| <i>Socs3</i> | 17 | 40.92 | 17 | 26.16 | 1.79E-11 | 0.4451 |
| <i>Fpr1</i> | 18 | 38.41 | 26 | 21.24 | 2.66E-09 | 0.8110 |
| <i>Tnfaip3</i> | 19 | 36.91 | 20 | 24.25 | 6.30E-09 | 0.4238 |
| <i>Cxcl2</i> | 20 | 36.81 | 7 | 60.84 | 6.79E-14 | 0.9650 |
| <i>BB548587</i> | 21 | 36.54 | 71 | 11.55 | 1.85E-06 | 0.7213 |
| <i>Tnfsf9</i> | 22 | 35.17 | 64 | 12.63 | 5.17E-08 | 0.9962 |
| <i>Socs3</i> | 23 | 35.10 | 18 | 25.02 | 1.24E-12 | 0.2296 |
| <i>Ccl2</i> | 24 | 34.01 | 21 | 23.20 | 1.41E-09 | 0.5926 |
| <i>Ccl7</i> | 25 | 33.98 | 9 | 42.02 | 8.91E-10 | 0.3433 |
| <i>Fpr-rs2</i> | 26 | 31.15 | 27 | 21.21 | 3.19E-12 | 0.6983 |
| <i>Il1rn</i> | 27 | 29.49 | 69 | 11.71 | 2.74E-10 | 0.0624 |
| <i>Ets2</i> | 28 | 29.25 | 49 | 15.50 | 3.77E-09 | 0.5326 |
| <i>Ccrl2</i> | 29 | 29.13 | 30 | 19.91 | 2.75E-08 | 0.7250 |
| <i>LOC622976</i> | 30 | 28.83 | 12 | 33.45 | 6.43E-12 | 0.1185 |
| <i>Il1rn</i> | 31 | 28.08 | 32 | 18.99 | 4.52E-11 | 0.1198 |
| <i>Tnfaip3</i> | 32 | 27.38 | 24 | 21.82 | 1.42E-10 | 0.8902 |
| <i>Zc3h12c</i> | 33 | 25.12 | 23 | 22.49 | 7.12E-10 | 0.3216 |
| <i>Ccl12</i> | 34 | 25.12 | 59 | 13.15 | 1.25E-08 | 0.2671 |
| <i>Cdc42ep2</i> | 35 | 24.16 | 44 | 16.31 | 1.03E-08 | 0.7966 |
| <i>Gpr84</i> | 36 | 23.46 | 31 | 19.54 | 3.15E-10 | 0.2886 |
| <i>Il1rn</i> | 37 | 23.35 | 73 | 11.35 | 9.55E-12 | 0.1113 |
| <i>Nr4a3</i> | 38 | 22.46 | 48 | 15.73 | 2.80E-08 | 0.7223 |
| <i>Ccl5</i> | 39 | 22.15 | 36 | 18.02 | 1.01E-07 | 0.2819 |
| <i>Ch25h</i> | 40 | 21.83 | 65 | 12.59 | 9.66E-11 | 0.2134 |
| <i>Cd40</i> | 41 | 21.42 | 66 | 12.25 | 2.74E-11 | 0.8623 |
| <i>Cd40</i> | 42 | 21.31 | 54 | 13.89 | 6.89E-12 | 0.3615 |
| <i>Zc3h12c</i> | 43 | 21.07 | 28 | 21.05 | 3.52E-10 | 0.7250 |
| <i>Clec4e</i> | 44 | 20.75 | 29 | 20.40 | 2.51E-09 | 0.9522 |
| <i>Malt1</i> | 45 | 20.22 | 33 | 18.56 | 5.46E-12 | 0.8940 |
| <i>Hivep3</i> | 46 | 19.93 | 22 | 22.60 | 3.95E-15 | 0.4636 |
| <i>Ptges</i> | 47 | 19.70 | 53 | 14.23 | 2.23E-10 | 0.2554 |
| <i>Cd69</i> | 48 | 19.60 | 34 | 18.55 | 3.57E-07 | 0.5920 |
| <i>Cd40</i> | 49 | 19.48 | 60 | 13.08 | 2.69E-12 | 0.7930 |
| <i>Traf1</i> | 50 | 19.18 | 43 | 16.55 | 5.61E-08 | 0.2824 |

Probe sets from the top 50 induced targets in PnCW-stimulated control cells are ordered 1 through 50 based on fold induction relative to the untreated control cells at 4 h. The correlative rank of genes expressed in PnCW-stimulated NOD2-deficient macrophages relative to the control ranking is listed along with the *P*-values (ANOVA) by time relative to unstimulated cells and by genotype.

pro-inflammatory genes induced by PnCW were dependent on TLR2 (Fig. 3), but independent of NOD2 as shown by the microarray data (Table 1). These results are consistent with the notion that TLR2 is the primary pathway by which macrophages detect PnCW and that NOD2 plays a downstream role in fine tuning the signalling response, in this case by regulating IL-10 production.

NOD2 has been reported to form heterotypic complexes with another CARD domain protein, RIPK2

(Kobayashi *et al.*, 2002; Park *et al.*, 2007; Hasegawa *et al.*, 2008). Like NOD2-deficient cells, macrophages lacking RIPK2 cannot respond to MDP, suggesting that NOD2 and RIPK2 function in the same pathway for MDP responsiveness (Park *et al.*, 2007). Furthermore, RIPK2 is also required for NOD1 function (which does not 'sense' MDP), suggesting that RIPK2 is genetically and biochemically linked to both NOD1 and NOD2 activity (Park *et al.*, 2007). We therefore used macrophages derived from two

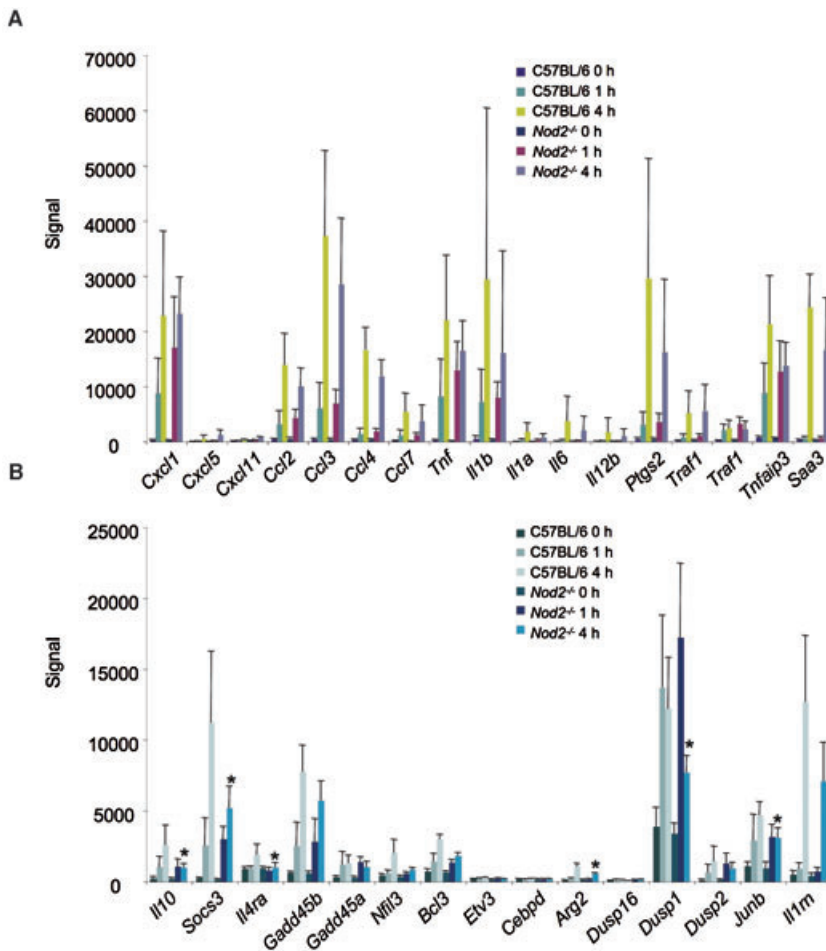


Fig. 2. Absence of NOD2 has a limited effect on PnCW-induced inflammatory transcription. A. Select inflammatory targets induced by PnCW in control or NOD2-deficient BMDMs. Data for each target mRNA is plotted as the average difference signal intensity. Data are averages from independent ($n = 4$) Affymetrix arrays performed per genotype and per time. No significant differences (paired t -test) were detected between genotypes. Complete data sets for this experiment have been deposited in the GEO database.

B. Expression of IL-10 target genes is affected in the absence of NOD2 function. Signal intensity for IL-10 (left most in the panel) and selected genes whose expression is dependent, in part, on autocrine-paracrine IL-10 production. * $P < 0.05$, paired t -test comparing 4 h time points between control and $Nod2^{-/-}$ BMDMs ($n = 4$).

strains of $Ripk2^{-/-}$ mice and mice lacking both NOD1 and NOD2 ($Nod1^{-/-};Nod2^{-/-}$) to probe if IL-10 production was inhibited. We observed by ELISA and qRT-PCR analysis that RIPK2-deficient cells had a similar phenotype to NOD2-deficient BMDMs (Fig. 4) in terms of reduced IL-10 production in response to PnCW. Furthermore, macrophages from $Nod1^{-/-};Nod2^{-/-}$ mice, which have been reported to have similar phenotypes to $Ripk2^{-/-}$ macrophages (Park *et al.*, 2007), also had a deficiency in IL-10 production when stimulated with PnCW. Finally, when introduced into $Nod2^{-/-}$ macrophages by retroviral-mediated transduction, human NOD2 rescued IL-10 in response to PnCW and MDP responsiveness (Fig. 4E–G). Analogous studies with NOD1 were not possible because human NOD1 producer lines do not make virus at sufficiently high titre to infect stem cells. Collectively, these data provide evidence that NOD2 and RIPK2 function in the same pathway that mediated IL-10 production in response to PnCW.

Zymosan, a complex cell wall fraction from yeast, has been shown to induce IL-10 production by the TLR2-DECTIN1-SYK pathway and the TLR2-DECTIN1-ERK pathway (Dillon *et al.*, 2004; 2006; Rogers *et al.*, 2005;

Slack *et al.*, 2007). We therefore asked if PnCW also stimulated ERK activation with the idea that ERK could be a common downstream mediator of multiple TLR2-driven pathways, including the TLR2-NOD2 pathway. However, we observed that PnCW induced low or negligible ERK phosphorylation (Fig. 5). Therefore, PnCW seems to stimulate IL-10 production via pathways distinct from those induced by zymosan, even though both the DECTIN1-SYK and NOD2-RIPK2 pathways depend on TLR2. Finally, IL-10 production in response to PnCW was dependent on MyD88 (Fig. 5E), suggesting that the TLR2-MyD88 pathway is absolutely necessary for PnCW-induced IL-10 production. MyD88 was not, however, required for MDP sensing (Fig. 5F) as expected as shown by the use of a sensitive MDP stimulation assay where nitric oxide production is measured in response to MDP and IFN- γ (Totemeyer *et al.*, 2006). By contrast, MDP sensing was dependent on NOD2.

Discussion

Our data suggest that NOD2 is a component of a signalling module that regulates IL-10 production in a

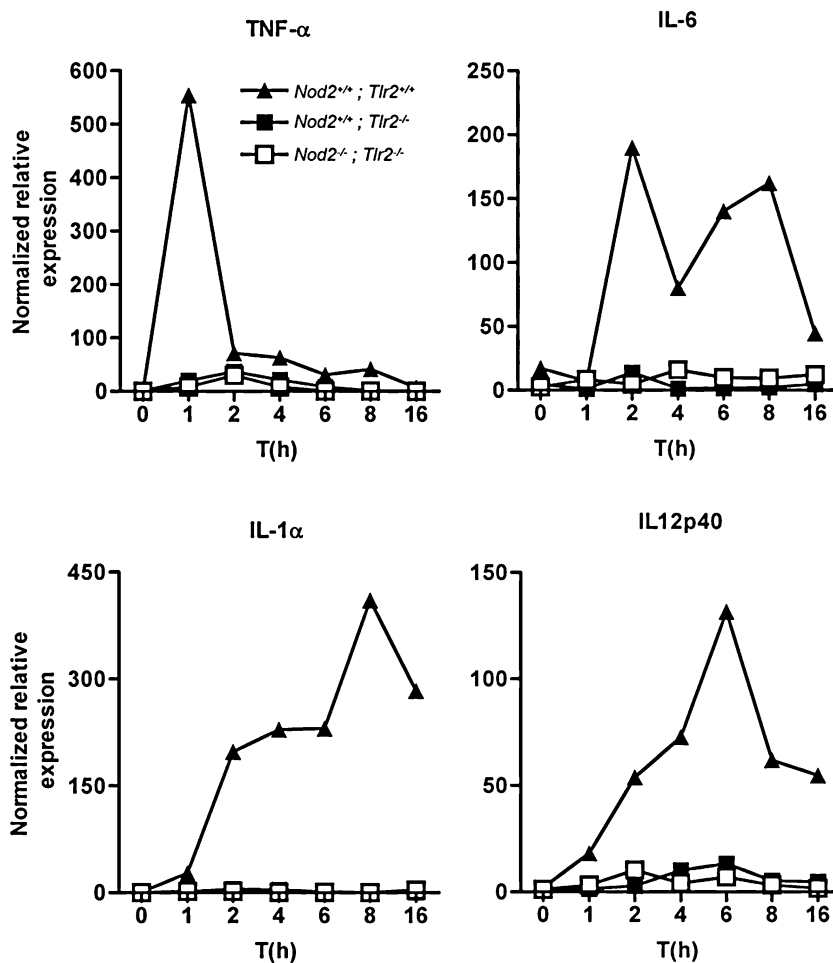


Fig. 3. Pro-inflammatory cytokine transcription in response to PnCW is dependent on TLR2. BMDMs from the indicated genotypes were stimulated with PnCW for the times shown. Cytokine mRNAs were measured by qRT-PCR. Data are representative of three independent experiments.

stimulus-specific and cell type-specific way (Fig. 6). Activation of TLR2-MyD88 by Pam₃CSK4 leads to ERK activation and IL-10 induction. In contrast, activation of TLR2-MyD88 by PnCW invokes NOD2 and RIPK2 and bypasses ERK activation to produce IL-10. Activation of TLR2 and DECTIN1 by zymosan leads to ERK activation and IL-10 induction. It is well accepted that IL-10 is essential for regulating the amplitude of most, if not all inflammatory responses *in vivo*. Therefore, mutant forms of NOD2 may be a component of homeostatic regulation of IL-10 in myeloid-derived cells. Notably, Netea and colleagues have demonstrated that human monocyte-derived macrophages and dendritic cells isolated from people bearing NOD2 mutations have reduced IL-10 production in response to peptidoglycan and MDP (Netea *et al.*, 2004; 2005; Kullberg *et al.*, 2008).

We found parallels between PnCW stimulation of IL-10 production and the activation of dendritic cells by zymosan, a large fragmentary remnant of yeast cell walls. In both cases, immune cells are exposed to particulate fractions of cell walls of varying sizes and displaying a variety of different sugars, lipids and structural

biopolymers. Zymosan stimulates IL-10 production by a MyD88-independent pathway involving TLR2, DECTIN1, SYK and ERK (Dillon *et al.*, 2004; 2006; Rogers *et al.*, 2005; Slack *et al.*, 2007). PnCW stimulation of IL-10 also requires TLR2, but does not seem to activate substantial ERK phosphorylation (Fig. 5). Instead, MyD88, NOD2 and RIPK2 are required. The PnCW and zymosan recognition pathways present a considerable contrast to the activation of immune cells with Pam₃CSK4 through TLR2, a pathway that has an absolute requirement for MyD88 but not NOD2. Therefore, there are different signalling routes to IL-10 production following TLR2 activation (Fig. 6). Similarly, additional diversity of signalling for IL-10 production has been observed for receptors such as the LPS-mediated pathway that requires TLR4 and p38 (Park *et al.*, 2005), and the immune complex pathway that enhances IL-10 production via FcR ligation (Edwards *et al.*, 2006). BTK also plays a key role in stimulus-specific IL-10 production: the absence of BTK leads to a decrease in IL-10 produced from macrophages and a corresponding increase in inflammatory mediators normally blocked by IL-10 (e.g. IL-12, IL-6) (Kawakami *et al.*, 2006; Schmidt

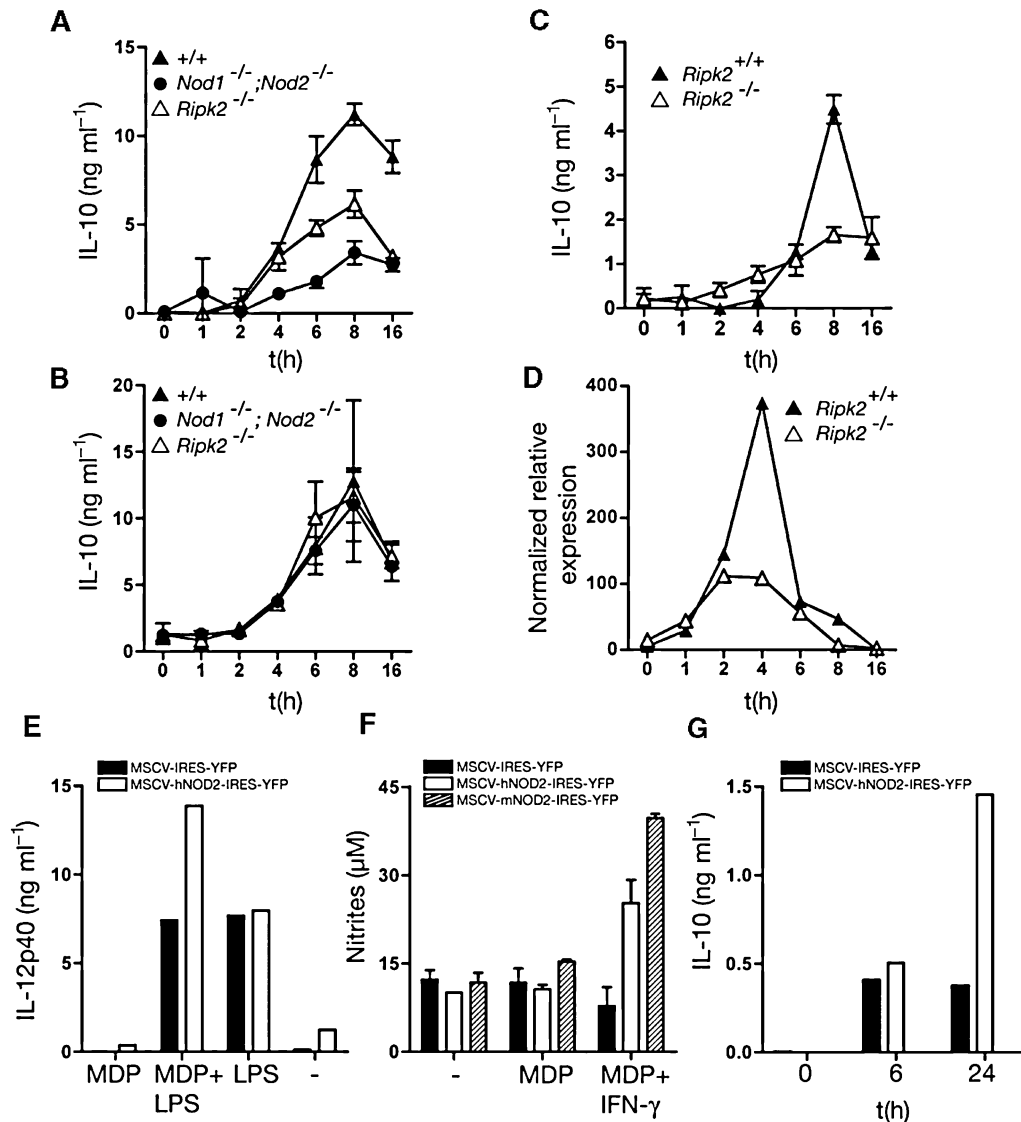


Fig. 4. IL-10 expression in response to PnCW is dependent on the RIPK2 pathway.

A. BMDMs from *Ripk2*^{+/+} or *Nod1*^{-/-}; *Nod2*^{-/-} mice were stimulated with PnCW and IL-10 measured over time by ELISA.

B. As in (A), but following LPS stimulation.

C and D. Analysis of BMDMs from an independent *Ripk2* mutant strain showing IL-10 and IL-10 mRNA produced in response to PnCW requires RIPK2.

E and F. Reconstitution of *Nod2*^{-/-} hematopoietic stem cells with human or mouse NOD2 cDNAs rescues IL-10 production in response to PnCW. Stem cells from *Nod2*^{-/-} mice were infected with retroviruses containing hNOD2 or mNOD2 cDNAs and selected by cell sorting for YFP and plated into media containing CSF-1 to drive differentiation into macrophages. After differentiation, macrophages were stimulated with LPS or LPS and MDP to measure the MDP synergy with TLR4 signalling (E) or IFN-γ signalling (F) or IL-10 production after PnCW treatment (G). Data are representation of two independent infection studies. In the case of mNOD2, sufficient cells were obtained to perform the MDP-IFN-γ synergy assay only.

et al., 2006). However, the reduced amounts of IL-10 made in the absence of BTK, like the absence of NOD2, is insufficient to initiate the severe inflammatory bowel disease observed in the complete absence of IL-10 (Schmidt *et al.*, 2006) (L.M. and P.J.M., unpubl. data). Therefore, NOD2 and BTK both seem to function to tune stimulus-specific IL-10 production.

We found that neither TLR2 nor NOD2 were absolutely required for PnCW stimulation of IL-10 production. IL-10

was made, although in much lower amounts, in the absence of either TLR2 or NOD2. However, cells lacking both TLR2 and NOD2 had a complete abrogation of IL-10 production. These data suggest that TLR2 and NOD2 have a genetic interaction. A similar finding has been made in the ECOVA gut inflammation system where pathology observed in the absence of NOD2 was reversed in the combined deficiency of both NOD2 and TLR2 (Watanabe *et al.*, 2006). It is however, premature to

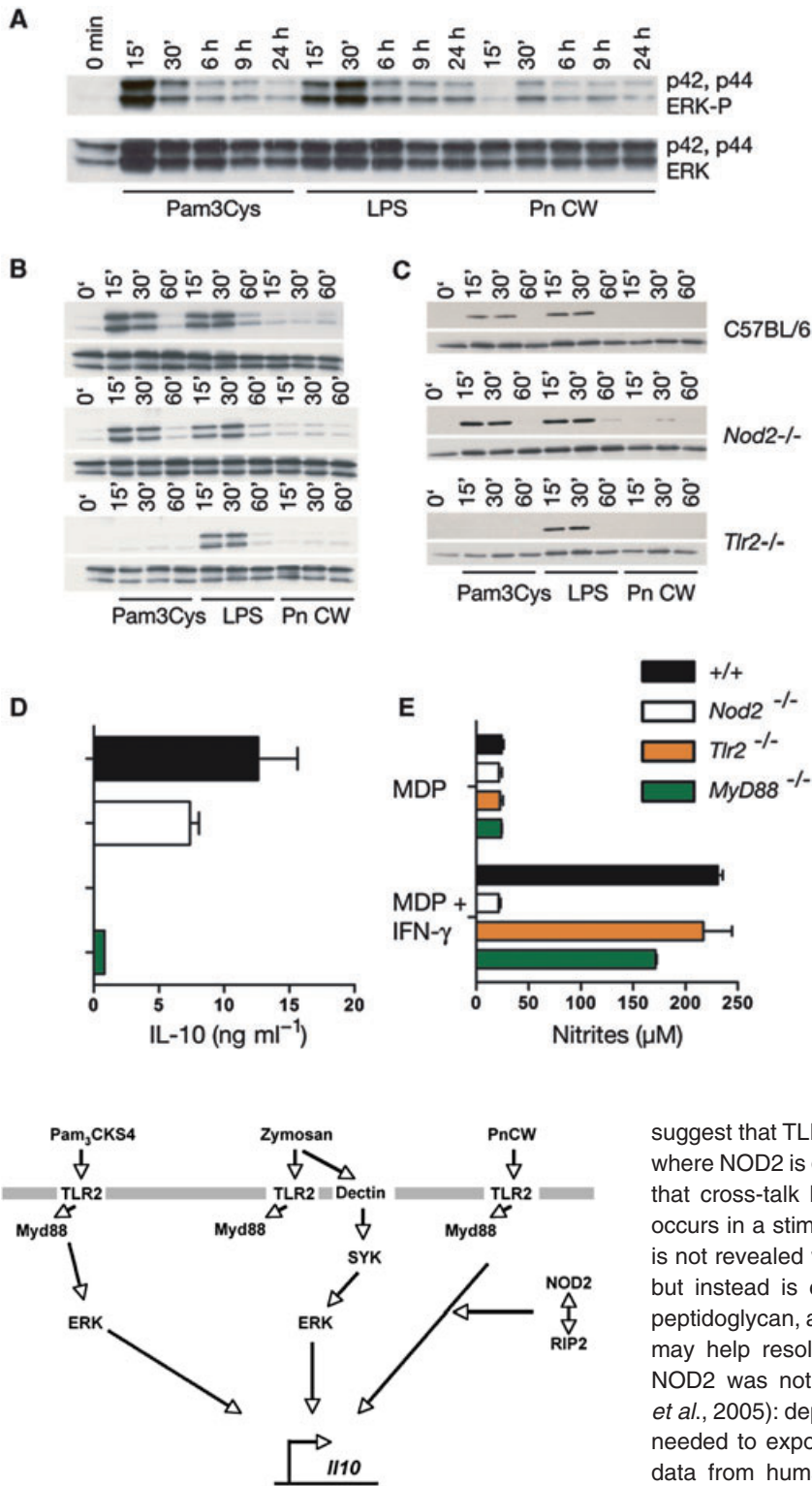


Fig. 5. PnCW activation of BMDMs does not activate the ERK or p38 pathways but requires MyD88 signalling.

A. C57BL/6 BMDMs were stimulated with Pam₃CSK4, LPS or PnCW over time and ERK phosphorylation measured by immunoblotting for phosphorylated forms of ERK or reprobbed for total ERK amounts.

B. A similar experiment to (A) was performed using BMDMs from the genotypes indicated on the right.

C. The same lysates from (B) were probed for phospho-p38. Note that PnCW does not activate ERK or p38 in any of these experiments.

D. BMDMs from control, *Nod2*^{-/-}, *Tlr2*^{-/-} or *MyD88*^{-/-} mice were stimulated with PnCW for 8 h and IL-10 measured by ELISA. Data are average ± SD from independent samples (*n* = 3).

E. BMDMs were stimulated with MDP or MDP + IFN-γ overnight and nitrites measured by the Griess assay. Stimulation with IFN-γ alone did not produce detectable nitrites (data not shown).

Fig. 6. Model of IL-10 production myeloid lineage cells (macrophages and DCs) stimulated by agents that activate TLR2, alone or in combination with other cell surface molecules.

suggest that TLR2 and NOD2 function in a linear pathway where NOD2 is downstream of TLR2. Instead, we suggest that cross-talk between the TLR2 and NOD2 pathways occurs in a stimulus- and cell-specific context. Cross-talk is not revealed when TLR2 is stimulated with Pam₃CSK4 but instead is exposed by agents such as PnCW and peptidoglycan, at least in the BMDMs used here. This idea may help resolve previous reports that suggested that NOD2 was not involved in TLR2 signalling (Kobayashi *et al.*, 2005): dependence on both stimulus and cell type is needed to expose differences among pathways. Recent data from human monocytes bearing *NOD2* mutations has also revealed a complex interplay between NOD2 and TLR2 (Borm *et al.*, 2008).

Using transcriptome profiling, we also found that the absence of NOD2 affected a surprisingly low number of mRNAs when BMDMs were stimulated with PnCW. These data are in keeping with published studies suggesting that

loss of NOD2 has minimal effects on pro-inflammatory TLR signalling (Pauleau and Murray, 2003; Kobayashi *et al.*, 2005; Park *et al.*, 2007). However, PnCW is an example of a pathogen 'signal' that can activate multiple pathways including the TLR2 pathway. It is not surprising that NOD2 plays limited roles in the overall transcriptional response as the mammalian immune system most likely has developed multiple, overlapping ways to recognize and respond to such a complex material. Nevertheless, the finding that IL-10 was one of the most affected mRNAs, along with a cohort of IL-10-regulated genes, suggests that further work is warranted to determine the role of NOD2 in IL-10 production in humans bearing mutations in *NOD2*.

It is tempting to speculate that IL-10 regulation has key functional significance in chronic inflammatory states such as the inflammatory bowel diseases. Most researchers agree that common pathways of inflammatory mediator production, arrived at by a multitude of genetic and environmental mechanisms, drive disease and are the central focus of therapeutic intervention through anticytokine antagonists. IL-10 plays an essential regulatory role in controlling the products of the common inflammatory pathway: IL-12, TNF- α , IL-6, etc. Therefore, small disturbances in temporal, anatomic or quantitative IL-10 amounts may translate into greater inflammation.

Experimental procedures

Mice

Nod2^{-/-} mice have been previously described (Pauleau and Murray, 2003). *Nod2*^{-/-};*Tlr2*^{-/-} mice on a C57BL/6 background (*n* = 5 generations) have been described (Watanabe *et al.*, 2006). *Tlr2*^{-/-} mice were purchased from the Jackson Laboratories. Mice were bred and genotyped according to published protocols. For some experiments, *Nod2*^{-/-} mice were backcrossed 10 generations to the C57BL/6 background. All animal experiments were performed in accordance with protocols governed by the St Jude Animal Care and Use Committee (P.J. M., Principal Investigator). *Nod1*^{-/-};*Nod2*^{-/-} and *Rip2k*^{-/-} mice have been described previously (Chin *et al.*, 2002; Kobayashi *et al.*, 2002; Park *et al.*, 2007).

Cell preparation and stimulus conditions

Bone marrow-derived macrophages were isolated and cultured as described (Lang *et al.*, 2002). Macrophages were stimulated with highly purified PnCW prepared as described previously (Tuomanen *et al.*, 1985a,b). Briefly, *S. pneumoniae* unencapsulated strain R6 were grown in C+Y medium, bacteria were boiled in SDS, mechanically broken by shaking with acid-washed glass beads, and treated sequentially with DNase, RNase, trypsin, LiCl, EDTA and acetone. PnCW was confirmed to be free of protein by analysis for non-cell wall amino acids using mass spectrometry. The absence of contaminating endotoxin was confirmed first by the *Limulus* test (Associates of Cape Cod) and then by ELISA

measurement of hIL-8 production by HEK 293 cells transfected with plasmids designed to express TLR4 and MD2 (a generous gift of Dr Doug Golenbock, UMass) in the presence of purified PnCW. For fluorescence microscopy experiments, BMDMs were labelled with Phalloidin and 4',6-diamidino-3-phenylindole (DAPI), a nuclear stain. PnCW was directly labelled with 1 mg ml⁻¹ FITC (Sigma-Aldrich) solution in carbonate buffer (pH 9.2) for 1 h at room temperature in the dark and washed twice with PBS containing calcium and magnesium (Gosink *et al.*, 2000).

ELISA

ELISA was performed as previously described (El Kasmi *et al.*, 2006). Capture and detection antibodies used were purchased from BD Pharmingen. Detection limits for the ELISAs were 50 pg ml⁻¹ (IL-10) and 10 pg ml⁻¹ (IL-12p40).

Northern blotting, RT-PCR and immunoblotting

Total RNA was isolated from primary macrophages using Trizol. Reverse transcription (qRT-PCR) was performed as described previously using primers and probes (Applied Biosciences) specific for each cytokine mRNA (Lang *et al.*, 2002). Immunoblotting was performed using the following rabbit polyclonal antibodies: antiphospho-p42, p44 ERK and anti-phospho-p38 from Cell Signaling Technology used at 1:500 final dilution. Anti-p42, p44 ERK and anti-p38 were from the same source and were used at a final concentration of 1:1000.

Transcriptome analysis

RNA samples from BMDM cultures representing four PnCW preparations, two genotypes (wild-type and the NOD2 deficient) and three time points (0, 1 and 4 h) were arrayed on Affymetrix GeneChip analysis murine 430v2GeneChip arrays. Signal was acquired with MAS5.0 software and natural log transformed [ln(signal + 20)] to stabilize variance and better conform the data to the normal distribution. Principal components analysis (Partek 6.1) revealed a batch effect which, being orthogonal to hour and genotype, was removed by capturing and mean adjusting residuals of a one-way ANOVA-based on PnCW batch (STATA/SE 9.2). The batch-corrected transformed signal was tested for exposure time and genotype effects in a two-way ANOVA using Partek. The *P*-values for the time effect were corrected for multiple comparisons using the false discovery rate (Benjamini *et al.*, 2001). Fold change calculations were based on the geometric means of the batch corrected data between the 4 h and 0 h time points for each genotype. Array data has been submitted to the GEO database (accession GSE8960).

Retroviral-mediated transduction of stem cells

Retroviral transduction of murine bone marrow cells was performed as described previously (El Kasmi *et al.*, 2006; Holst *et al.*, 2006a,b). For the transduction experiments, we used fetal liver stem cells isolated from E14 pregnant *Nod2*^{-/-} female mice. Fetal liver cells were cultured for 48 h in complete DMEM with 20% FBS, 20 ng ml⁻¹ murine IL-3, 50 ng ml⁻¹ human IL-6 and

50 ng ml⁻¹ murine stem cell factor (BioSource International). Stem cells were subsequently co-cultured for a further 48 h with irradiated (1200 rad) retroviral producer cell lines plus 6 mg ml⁻¹ polybrene and cytokines as previously detailed. Non-adherent, transduced cells were collected, red and re-suspended in PBS with 2% FBS with 20 U ml⁻¹ heparin. Cells were sorted by gating on forward and side scatter, and YFP positivity. Cells were subsequently plated out with 500 ng ml⁻¹ Fungizone and 25 mg ml⁻¹ gentamicin in complete DMEM with 20% FBS and L cell conditioned medium as a source of CSF-1. Following differentiation, mature cells were measured by flow cytometry for YFP along with surface markers (CD11b, F4/80) for macrophages.

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