

Murine Nod1 but not its human orthologue mediates innate immune detection of tracheal cytotoxin

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Tracheal cytotoxin (TCT) was originally described as the minimal effector that was able to reproduce the cytotoxic response of *Bordetella pertussis* on ciliated epithelial cells. This molecule triggers pleiotropic effects such as immune stimulation or slow-wave sleep modulation. Further characterization identified TCT as a specific diaminopimelic acid (DAP)-containing muropeptide, GlcNAc-(anhydro)MurNAc-L-Ala-D-Glu-mesoDAP-D-Ala. Here, we show that the biological activity of TCT depends on Nod1, an intracellular sensor of bacterial peptidoglycan. However, Nod1-dependent detection of TCT was found to be host specific, as human Nod1 (hNod1) poorly detected TCT, whereas mouse Nod1 (mNod1) did so efficiently. More generally, hNod1 required a tripeptide (L-Ala-D-Glu-mesoDAP) for efficient sensing of peptidoglycan, whereas mNod1 detected a tetrapeptide structure (L-Ala-D-Glu-mesoDAP-D-Ala). In murine macrophages, TCT stimulated cytokine secretion and NO production through Nod1. Finally, *in vivo*, injection of the tetrapeptide structure in mice triggered a transient yet strong release of cytokines into the bloodstream and the maturation of macrophages, in a

Nod1-dependent manner. This study thereby identifies Nod1 as the long sought after sensor of TCT in mammals.

Keywords: Nod proteins; innate immunity; peptidoglycan

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INTRODUCTION

Nod1 and Nod2 are cytosolic pattern-recognition molecules involved in early detection of bacteria, resulting in the induction of pro-inflammatory mediators (Girardin & Philpott, 2004; Inohara *et al*, 2005). Both Nod molecules detect distinct muropeptide monomers from bacterial peptidoglycan. Nod2 is a general sensor for both Gram-positive and Gram-negative bacteria, as biochemical and functional analyses have identified muramyl dipeptide (MDP), the minimal motif common to all peptidoglycans, as the essential structure recognized by Nod2 (Girardin *et al*, 2003b; Inohara *et al*, 2003). In contrast, Nod1 presents a strict sensing specificity towards diaminopimelic acid (DAP)-type peptidoglycan; indeed, human Nod1 (hNod1) detects a single muropeptide, GM-Tri_{DAP}, which is produced as a peptidoglycan degradation product in Gram-negative bacterial metabolism (Chamaillard *et al*, 2003; Girardin *et al*, 2003a,c).

Tracheal cytotoxin (TCT) was originally defined as a product released by *Bordetella pertussis* that was able to recapitulate the toxicity of the bacterium on ciliated tracheal epithelial cells (Goldman *et al*, 1982). TCT was later found to be identical to GlcNAc-(anhydro)MurNAc-L-Ala-D-Glu-mesoDAP-D-Ala, a specific muropeptide present within the peptidoglycan of Gram-negative bacteria (Rosenthal *et al*, 1987). TCT was shown to induce a broad spectrum of biological responses on hamster tracheal epithelial cells, such as cell proliferation, NO synthesis and interleukin (IL)-1 secretion (Cookson *et al*, 1989). Recently, TCT was shown to trigger innate immunity in *Drosophila*, through the activation of peptidoglycan recognition protein LC (PGRP-LC), leading to the induction of the immune deficiency pathway

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(Kaneko *et al*, 2004; Stenbak *et al*, 2004). However, the sensor of TCT responsible for eliciting these biological responses in mammals remains unidentified.

We evaluated the ability of several DAP-containing tetrapeptide mucopeptides (TCT, M-Tetra_{DAP}, Lactyl-Tetra_{DAP} and FK156; see supplementary Fig S1 online) to activate hNod1 or hNod2, using the previously described co-transfection procedure in human epithelial cells (Inohara *et al*, 2001). These molecules behaved as poor activators of hNod1 compared with M-Tri_{DAP}, the previously identified Nod1 agonist; however, at the highest dose tested (250 nM), M-Tetra_{DAP}, Lactyl-Tetra_{DAP} and FK156 induced activation of hNod1 (Fig 1A). None of these structures could stimulate hNod2, even at the highest dose used (250 nM), whereas MDP, the well-defined agonist of Nod2, did so even at 10 nM (Fig 1B). In human peripheral blood mononuclear cells (PBMCs), mucopeptides that are added extracellularly can stimulate cytokine release, without the need for any liposomal carrier. In agreement with the results obtained in human embryonic kidney (HEK)293 cells, we observed that MDP and M-Tri_{DAP} stimulated tumour necrosis factor- α (TNF- α) secretion from human PBMCs, whereas the DAP-containing tetrapeptide mucopeptides (TCT, M-Tetra_{DAP} and Lactyl-Tetra_{DAP}) showed only marginal stimulatory activity (Fig 1C). Similar results were obtained for IL-8 (data not shown).

We next stimulated thioglycolate-elicited mouse peritoneal macrophages from wild-type (WT), Nod1^{-/-} or Nod2^{-/-} animals with mucopeptides (MDP, M-Tri_{DAP}, Lactyl-Tetra_{DAP} and TCT) or Toll-like receptor (TLR) agonists (lipopolysaccharide (LPS) for TLR4; Pam3Cys4-OH for TLR2) as positive controls. First, we observed that TLR-dependent stimulation of TNF- α secretion by LPS and Pam3Cys4-OH was both Nod1 and Nod2 independent (Fig 2A,B). In addition, stimulation of TNF- α secretion by MDP was fully mediated by Nod2 (Fig 2B) but not by Nod1 (Fig 2A). Conversely, Lactyl-Tetra_{DAP} and TCT could activate mouse macrophages in a Nod1-dependent manner (Fig 2A), whereas Nod2 was shown to be largely dispensable (Fig 2B). A dose-response experiment that showed increasing concentrations of either MDP or TCT further implied that Nod1 and Nod2 could, for the most part, function, if not fully independently (supplementary Fig S2 online). Surprisingly, we noticed that mouse peritoneal macrophages, although potently stimulated by Lactyl-Tetra_{DAP} and TCT, were poorly activated by M-Tri_{DAP} (Fig 2A,B). These findings are in sharp contrast to the results we obtained in human cells (Fig 1A–C). We therefore suggested that hNod1 and mouse Nod1 (mNod1) could possibly detect distinct mucopeptides. We compared the ability of MDP, DAP-containing tripeptide mucopeptides (M-Tri_{DAP} and Tri_{DAP}) and DAP-containing tetrapeptide mucopeptides (TCT, M-Tetra_{DAP}, Lactyl-Tetra_{DAP} and FK156) to activate hNod1 or mNod1 in *in vitro* transfection assays. hNod1 detected DAP-containing tripeptide mucopeptides, whereas mNod1 sensed these mucopeptides poorly (Fig 2C). Conversely, mNod1, but not hNod1, detected efficiently all DAP-containing tetrapeptide mucopeptides (Fig 2C). However, both hNod1 and mNod1 showed a lack of response towards MDP or Lys-type mucopeptides (M-Tri_{Lys} and M-Tetra_{Lys}; Fig 2D). Therefore, these results identify the length of the peptidic moiety of mucopeptides as a key determinant of peptidoglycan sensing by Nod proteins. In particular, when varying the length of the peptide from $n = 1$ to 5 amino acids (the only naturally occurring uncrosslinked

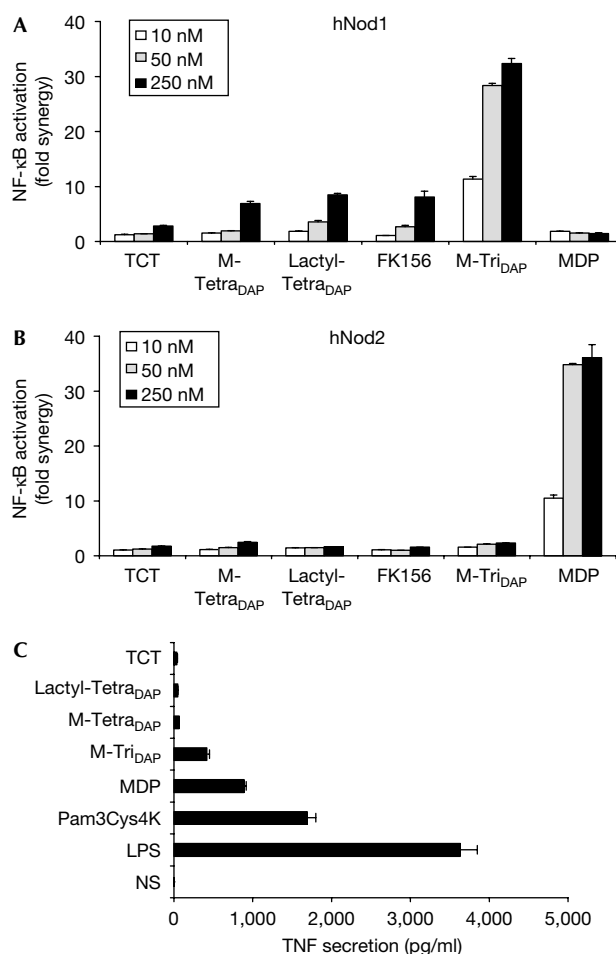


Fig 1 | Human Nod1 detects diaminopimelic acid-type muramyl tripeptides in both epithelial cells and immune cells. (A,B) Human embryonic kidney 293 epithelial cells were co-transfected with several muramyl peptides (tracheal cytotoxin (TCT), M-Tetra_{DAP}, Lactyl-Tetra_{DAP}, FK156, M-Tri_{DAP}, muramyl dipeptide (MDP)) at the concentrations 10 nM (white bars), 50 nM (grey bars) or 250 nM (black bars) in the presence of expression vectors for either human Nod1 (hNod1) (A) or hNod2 (B). In (A,B), the activity of a nuclear factor- κ B (NF- κ B)-driven luciferase reporter gene was measured, and Nod1-dependent (A) or Nod2-dependent (B) activation of the reporter gene in the presence of muramyl peptides was reported compared with that obtained without stimulation with muramyl peptides. Data are the mean \pm s.e.m. of duplicates and are representative of three independent experiments. (C) Human peripheral blood mononuclear cells from an individual donor were stimulated overnight with lipopolysaccharide (LPS; 10 ng/ml), Pam3Cys4-OH (Pam3Cys4K, 1.5 ng/ml) or a selection of muramyl peptides (MDP, M-Tri_{DAP}, TCT, M-Tetra_{DAP} and Lactyl-Tetra_{DAP}; all at 50 nM) and tumour necrosis factor- α (TNF- α) secretion was measured in the supernatant by enzyme-linked immunoabsorbent assay. NS, nonstimulated. Data shown are from a single donor and are representative of four independent experiments. Data are the mean \pm s.e.m. of duplicates.

mucopeptides), we observed the clear preference of each Nod protein tested (hNod1, mNod1 and hNod2) for a specific peptidic length (Fig 2E). Importantly, whereas activation of hNod1 and

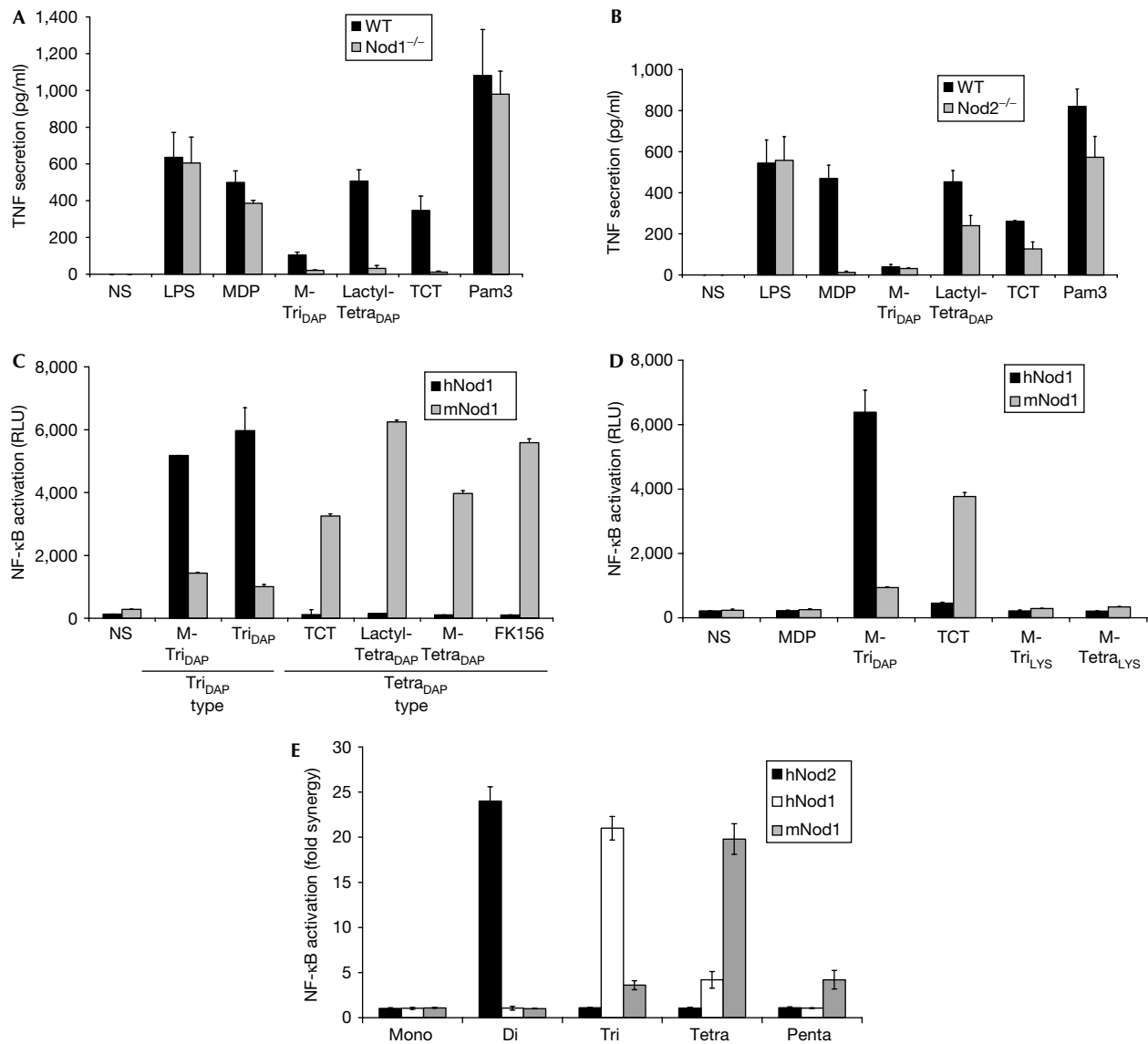


Fig 2 | Murine Nod1 preferentially detects diaminopimelic acid-type muramyl tetrapeptides. (A,B) Mouse peritoneal macrophages from C57Bl6 (wild type (WT)) and Nod1^{-/-} animals (A) or Nod2^{-/-} animals (B) were stimulated overnight with lipopolysaccharide (LPS; 10 ng/ml), Pam3Cys4-OH (Pam3, 1.5 ng/ml) or a selection of muramyl peptides (muramyl dipeptide (MDP), M-Tri_{DAP}, Lactyl-Tetra_{DAP} and tracheal cytotoxin (TCT); all at 250 nM) in the presence of 1 μM cytochalasin D to allow better stimulation by muramyl peptides, and tumour necrosis factor-α (TNF-α) secretion was measured by enzyme-linked immunoabsorbent assay. Data are the mean ± s.e.m. of duplicates and are representative of two independent experiments. (C,D) Human embryonic kidney (HEK)293 epithelial cells were transfected with muramyl peptides (M-Tri_{DAP}, Tri_{DAP}, TCT, FK156, Lactyl-Tetra_{DAP}, M-Tetra_{DAP}, M-Tri_{LYS}, M-Tetra_{LYS}; all at 50 nM) together with expression vectors for either human Nod1 (hNod1) or mouse Nod1 (mNod1), and nuclear factor-κB (NF-κB) activation was measured as in Fig 1A. NS, nonstimulated; RLU, relative light units. (E) HEK293 epithelial cells were transfected with 50 nM muramyl peptides (mono (MurNac-L-Ala), di (MDP), tri (M-Tri_{DAP}), tetra (M-Tetra_{DAP}) or penta (M-Penta_{DAP})) together with expression vectors for hNod2, hNod1 or mNod1. NF-κB activation was measured as in Fig 1A. For (C-E), data are the mean ± s.e.m. of duplicates and are representative of three independent experiments.

mNod1 shows a partial overlap in sensing preference (for $n=3$ and 4), neither of the Nod1 proteins shared any redundancy with Nod2 for muuropeptide detection (Fig 2E).

The identification of DAP-containing tetrapeptide muuropeptides as Nod1 agonists in mice allowed us to study directly the contribution of Nod1 in the modulation of innate immune responses. Mouse peritoneal macrophages from either WT or

Nod1^{-/-} mice were stimulated for 18 h with TCT, Lactyl-Tetra_{DAP} or LPS as a control, and cytokine secretion was measured. It is noteworthy that durations shorter than 18 h were not repeatedly investigated, as TCT stimulation for up to 8 h yielded marginal responses (supplementary Fig S3 online). Both TCT and Lactyl-Tetra_{DAP} induced Nod1-dependent secretion of TNF-α, IL-6, IL-1β and keratinocyte-derived cytokine (KC) (Fig 3A–D). LPS-induced

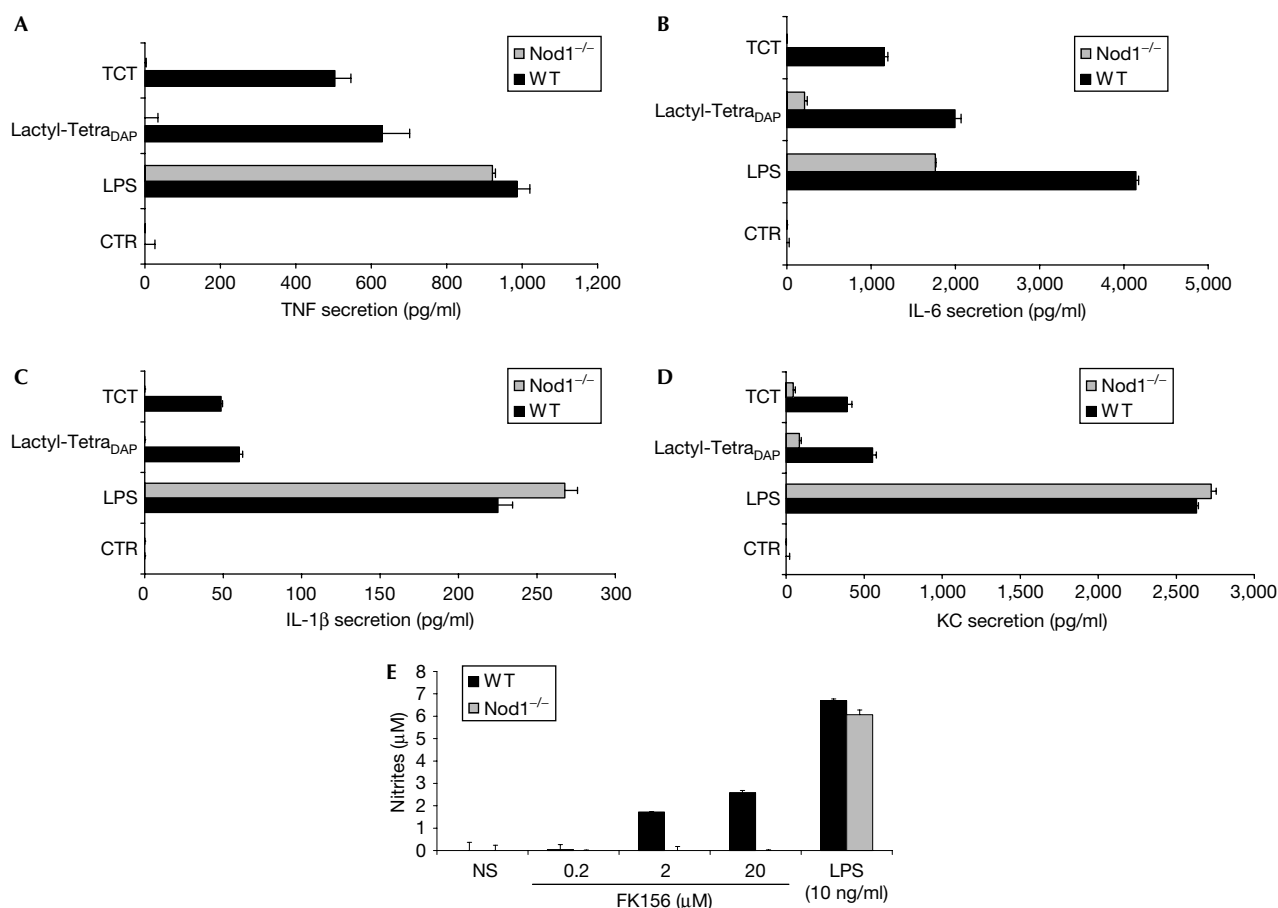


Fig 3 | Nod1 agonists induce cytokine and nitrite responses in mouse peritoneal macrophages. (A–D) Mouse peritoneal macrophages from either wild-type (WT) or Nod1^{-/-} animals were stimulated overnight with lipopolysaccharide (LPS; 10 ng/ml), Lactyl-Tetra_{DAP} (250 nM) or tracheal cytotoxin (TCT; 250 nM) in the presence of 1 μM cytochalasin D, and tumour necrosis factor-α (TNF-α) (A), interleukin (IL)-6 (B), IL-1β (C) and KC (D) in the supernatant were measured by enzyme-linked immunoabsorbent assay. Data are the mean ± s.e.m. of duplicates and are representative of two independent experiments. CTR, non-stimulated control. (E) Mouse peritoneal macrophages from either WT or Nod1^{-/-} animals were stimulated overnight with either LPS (10 ng/ml) or increasing concentrations of FK156, in the presence of 1 μM cytochalasin D, and interferon-γ (IFN-γ, 1 ng/ml) as indicated, and nitrite accumulation in the supernatant was measured using the Griess test. NS, nonstimulated. Data are the mean ± s.e.m. of duplicates and are representative of two independent experiments.

stimulation of cytokine secretion was Nod1 independent, with the exception of IL-6, for which we constantly observed a two- to threefold reduction of secretion in Nod1^{-/-} macrophages that were stimulated with various TLR ligands (Fig 3B; data not shown). Similarly, the stimulation of interferon-γ (IFN-γ)-primed (1 ng/ml overnight) peritoneal macrophages with FK156 induced a Nod1-dependent production of NO, as observed by the accumulation of nitrites in the culture medium (Fig 3E). These results thus indicate that Nod1 is required for TCT-induced biological responses in murine macrophages.

Intraperitoneal injection with 0.5 ml per C57Bl6 mouse of a 300 μM (equivalent to 4 mg/kg) or higher solution of FK156 resulted in a substantial release of KC in the bloodstream 2 h after injection (Fig 4A). Interestingly, injection with 0.5 ml of 3 mM FK156 per mouse resulted in a transient release (peaking at 2 h) of proinflammatory mediators KC, IL-6 and TNF (Fig 4B–D) in the bloodstream, while returning to nearly steady-state levels 24 h after injection or later. This response was Nod1 mediated, as no

such effect could be seen in Nod1^{-/-} animals. Finally, macrophages from either C57Bl6 or Nod1^{-/-} mice injected with FK156 (0.5 ml of 3 mM FK156 per mouse) were collected and analysed by flow cytometry 72 h after injection. Analysis of cell-surface expression of major histocompatibility complex (MHC) class II receptors identified a macrophage population that underwent maturation as a result of FK156 local injection (Fig 4E, left panel). Again, this response was found to be impaired in Nod1^{-/-} mice (Fig 4E, right panel). These results therefore show that sensing of FK156 by Nod1 contributes, *in vivo*, to the establishment of an immune response programme.

In this study, we have shown that mNod1 and hNod1 detect distinct mucopeptides. Whereas hNod1 preferentially senses DAP-containing tripeptide mucopeptides, mNod1 detects DAP-containing tetrapeptide mucopeptides, including TCT, a peptidoglycan fragment that has been known for more than two decades to trigger pleiotropic biological responses (Martin *et al*, 1984; Melly *et al*, 1984; Cookson *et al*, 1989; Burroughs *et al*, 1993; Flak *et al*,

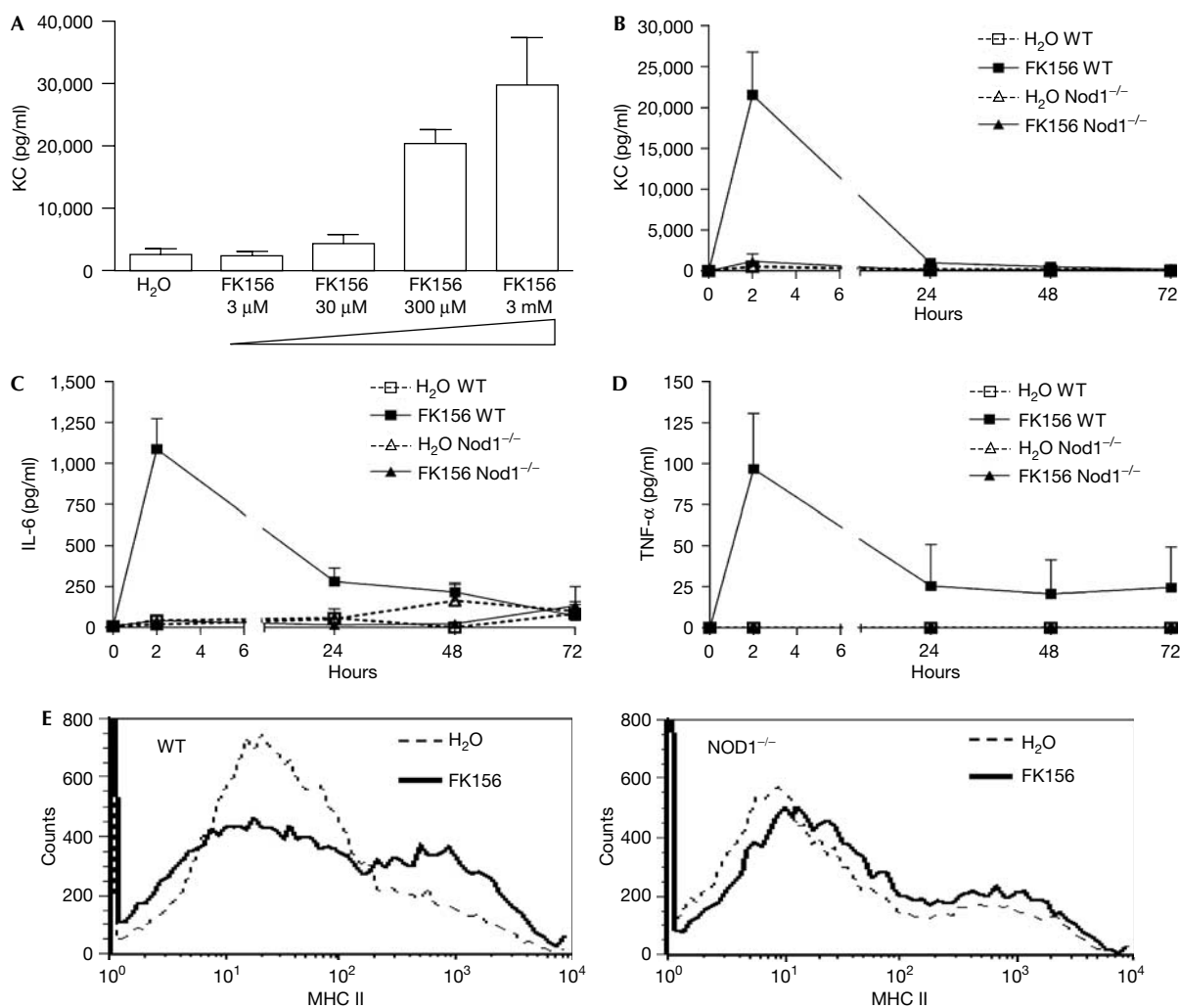


Fig 4 | Murine Nod1 agonist FK156 induces innate immune responses *in vivo* in mice. (A) Increasing concentrations of FK156 were given intraperitoneally (0.5 ml per C57Bl6 mouse). Blood was collected from animals 2 h after injection, and KC in the serum was measured by ELISA. (B–E) A 0.5 ml portion per mouse (C57Bl6 or Nod1^{-/-}) of 3 mM FK156 was given intraperitoneally and endotoxin-free water was injected as a control. KC (B), interleukin (IL)-6 (C) and tumour necrosis factor- α (TNF- α) (D) concentrations in the blood were recorded over time, as indicated, using enzyme-linked immunoabsorbent assay (ELISA). In another group of mice, peritoneal macrophages were isolated and analysed by flow cytometry for the cell-surface expression of major histocompatibility complex II (MHC II), a maturation marker, at their surface (E). WT, wild type.

2000). Our observations show that, in murine macrophages, Nod1 activates cytokine secretion and NO synthesis in response to TCT stimulation. In addition, the TCT analogue FK156 induces Nod1-dependent systemic responses *in vivo*, as well as maturation of macrophages at the site of injection. Consequently, Nod1 is likely to be a key mediator in the biological responses to DAP-containing tetrapeptide muropeptides, such as TCT.

Initial studies on TCT have shown that this molecule was able to recapitulate the cytotoxicity of a human pathogen, *B. pertussis*, on tracheal epithelial cells. However, most of these early studies have been carried out using cells from a heterologous host organism, the Syrian hamster (*Mesocricetus auratus*). From our results, we can speculate that cytotoxicity of *B. pertussis* occurs by TCT, not only in the Syrian hamster, but also in mice, and that in human cells, analogous biological effects would be mediated by

M-Tri_{DAP}. It remains possible, however, that an as yet unidentified receptor could detect TCT in specific subsets of human cells.

The characterization of distinct muropeptide requirements to achieve peptidoglycan sensing by hNod1 and mNod1 may have important implications in the study of some human pathogens that use mouse models of infection. Indeed, Gram-negative bacterial human pathogens such as *Salmonella*, *Shigella*, *Bordetella*, *Neisseria* or pathogenic *Escherichia coli* all have in common a high tetrapeptide/tripeptide ratio (up to 10/1) in their peptidoglycan (I.G. Boneca, Institut Pasteur, personal communication). From our results, we can anticipate that the innate immune responses to these pathogens might not be comparable in mice and humans, especially at the level of mucosal surfaces, in which Nod1 has been shown to have an important role (Girardin *et al*, 2003a; Chamailard *et al*, 2004; Kim *et al*, 2004).

METHODS

Muramyl peptides. The experimental procedures relative to the synthesis of all the muramyl peptides used in this study have been described elsewhere (Girardin *et al*, 2003c).

Reagents. Muramyl dipeptide (MDP-LD) was from Calbiochem (Fontenay sous bois, France) and was reported to be 98% pure by thin-layer chromatography. Synthetic FK156 was obtained from Fujisawa Inc. (Japan). Pam3Cys-Ser-Lys4-OH lipopeptide was from Roche Diagnostics (Mannheim, Germany). Pure LPS was a kind gift from Jean-Marc Cavaillon (Institut Pasteur, Paris, France). The absence of mucopeptide contamination in this LPS preparation has been demonstrated using co-transfection experiments with Nod1/Nod2 expression vectors in HEK293 cells, as described previously (Inohara *et al*, 2001). Cytochalasin D was from Sigma (Lyon, France). None of the reagents was found to significantly affect cell viability, as assessed by lactate dehydrogenase (LDH) assay (Promega, Madison, WI, USA), used according to the manufacturer's specifications.

Limulus amoebocyte assay. All reagents used tested negative for LPS contamination by the limulus amoebocyte assay, according to the manufacturer's recommendations (QCL-1000, BioWhittaker, Verviers, Belgium). These reagents include Pam3Cys4-OH, murine IFN- γ (mIFN- γ), cytochalasin D and all the muramyl peptides used in the study.

Expression plasmids and transient transfections. The expression plasmid for hNod1 was from Gabriel Nunez (Ann Arbor, MI, USA). The expression plasmid for hNod2 was from Gilles Thomas (Fondation Jean Dausset/CEPH, Paris, France). The expression plasmid for mNod1 was from Invivogen (Toulouse, France). The empty vector pcDNA3.1 and the nuclear factor- κ B (NF- κ B) reporter Igk luciferase were from Invitrogen. Transfections were carried out using FuGene (Roche, Neuilly sur Seine, France) in HEK293, as described previously (Girardin *et al*, 2003a).

Nuclear factor- κ B activation assays. HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Studies on the synergistic activation of NF- κ B by muramyl peptides were carried out as described previously (Inohara *et al*, 2001; Girardin *et al*, 2003a). NF- κ B-dependent luciferase assays were carried out in duplicate and data represent at least three independent experiments. Data are mean \pm s.e.m.

Mice. Mice (6–12 weeks old) were used for this study. C57BL6/J mice were purchased from Janvier (Le Genest, France). Nod1^{-/-} mice (Millenium Pharmaceuticals, CA, USA) have been backcrossed to the seventh generation into the C57BL6/J background at the Pasteur Institute. Mice were submitted to sanitary control tests at the CDTA (Orleans, France). All protocols were reviewed by the Institut Pasteur competent authority for compliance with the French and European regulations on Animal Welfare and Public Health Service recommendations. Nod2^{-/-} mice (from Marco Giovannini, CEPH, Paris) have been backcrossed to the seventh generation into the C57BL6/J background.

Cytokine dosage. Enzyme-linked immunoabsorbent assay (ELISA) for KC mIL-1 β , mTNF, hTNF, hIL-8, mIL-6 and mIL-10 were carried out according to the manufacturer's recommendations (DuoSet, R&D Systems Europe, Lille, France).

FK156 injection and flow cytometry. C57BL6 (WT) or Nod1^{-/-} mice (6–8 weeks old) were given 0.5 ml FK156 (or endotoxin-free water as a control) in the peritoneal cavity and blood was collected at various times at the tail vein (100 μ l per sample).

Serum was isolated from blood by fractionation and centrifugation (200g), and serum cytokines were measured by standard ELISA procedures. For flow cytometry, mice were injected with 0.5 ml of 3 mM FK156 and killed 3 days after injection. A 5 ml portion of saline was then injected in the peritoneal cavity and removed to collect intraperitoneal cells. Peritoneal cells were then incubated at 4 °C with antibodies for CD11b (BD Pharmingen, San Diego, CA, USA) and F4180 (eBioscience, Montrouge, France) to identify macrophages, MHC class II (BD Pharmingen), and samples were analysed using a FACScanto flow cytometer (BD Biosciences, Le Pont de Claix, France). Data were collected on approximately 30,000 cells.

Mouse peritoneal macrophages. Mouse peritoneal macrophages were elicited by injection of 1.5 ml of thioglycolate medium (Bio-Rad, Marne la Coquette, France) in the peritoneal cavity and isolated 4 days later, according to the procedures described previously (Travassos *et al*, 2004). In all experiments, cytochalasin D (1 μ M) was added directly to the cell culture medium 30 min before stimulation with TLR agonists or muramyl peptides, allowing increased stimulation by muramyl peptides. At the concentration used, cytochalasin D treatment for 18 h did not affect cell viability, as assessed by measurement of LDH release in the culture supernatant (data not shown). Also, the response to TLR agonists remained unaffected by this treatment (data not shown). After 18 h of stimulation (also, for the time-course experiments: 1, 2, 3 or 8 h of stimulation), the supernatants were aliquoted and frozen at -20 °C for subsequent cytokine dosage.

Peripheral blood mononuclear cell and monocyte isolation. PBMCs were prepared from fresh blood samples of healthy donors drawn on citrate/phosphate/dextrose (Etablissement Français du Sang, Paris, France), using the protocol described elsewhere (Fritz *et al*, 2005). PBMCs were stimulated with LPS, Pam3Cys4-OH or mucopeptides for 20 h before collection of supernatants.

Measurement of nitrite. Briefly, 100 μ l of the culture medium was incubated with 200 μ l of Griess reagent (1% sulphanilamide–0.1% naphthylethylene diamine dihydrochloride) at 20 °C for 30 min in the dark. The concentration of NO₂, a stable oxidized derivative of NO in cell cultures, was determined and the absorbance was measured at 540 nm using a Labsystem microplate reader. A calibration curve was prepared using sodium nitrite in the culture medium. The lower detection limit of this method was 1 μ M nitrite.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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