Trace levels of peptidoglycan in serum underlie the NOD-dependent cytokine response to endoplasmic reticulum stress

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Abstract
NOD1 and NOD2 are intracellular sensors of bacterial peptidoglycan that belong to the Nod-like receptor (NLR) family of innate immune proteins. In addition to their role as direct bacterial sensors, it was proposed that NOD proteins could detect endoplasmic reticulum (ER) stress induced by thapsigargin, an inhibitor of the sarcoplasmic or endoplasmic reticulum calcium ATPase family (SERCA) that pumps Ca^{2+} into the ER, resulting in pro-inflammatory signalling. Here, we confirm that thapsigargin induces NOD-dependent pro-inflammatory signalling in epithelial cells. However, the effect was specific to thapsigargin, as tunicamycin and the subtilase cytotoxin SubAB from Shiga toxigenic Escherichia coli, which induce ER stress by other mechanisms, did not induce cytokine expression. The calcium ionophore A23187 also induced NOD-dependent signalling, and calcium chelators demonstrated a role for both intracellular and extracellular calcium in mediating thapsigargin-induced and NOD-dependent pro-inflammatory signalling, in part through the activation of plasma membrane-associated calcium release-activated channels (CRACs). Moreover, our results demonstrate that both endocytosis and the addition of serum to the cell culture medium were required for thapsigargin-mediated NOD activation. Finally, we analyzed cell culture grade fetal calf serum as well as serum from laboratory mice by high-pressure liquid chromatography and mass spectrometry, and identified the presence of various peptidoglycan fragments. We propose that cellular perturbations that affect intracellular Ca^{2+} can trigger internalization of peptidoglycan trace contaminants found in culture serum, thereby stimulating pro-inflammatory signalling. The presence of peptidoglycan in animal serum suggests that a homeostatic function of NOD signalling may have been previously overlooked.

Introduction
Detection of microbes by the innate immune system relies on several families of pattern recognition molecules (PRMs) that recognize conserved microbe-associated molecular patterns (MAMPs) that are highly conserved and are not...
produced by the non-infected host. Among those families of PRMs are the Toll-like receptors (TLRs) and Nod-like receptors (NLRs). In addition to the detection of MAMPs, certain NLR proteins, such as NLRP3, detect cellular perturbations or molecules, known as danger-associated molecular patterns (DAMPs) that can arise as a result of an infection or following aseptic tissue damage (1).

NOD1 and NOD2 are two members of the NLR family of PRM that detect bacterial peptidoglycan (2). The specificity of NOD1 and NOD2 for peptidoglycan motifs is extremely high, as NOD1 detects meso-diaminopimelic acid-containing N-acetyl muramic acid (MurNAc)-tripeptide (Mur-TriDAP) found predominantly in Gram-negative bacteria (3-6), whereas NOD2 detects muramyl dipeptide (MDP) found in both Gram-negative and Gram-positive bacteria (3,7,8). Detailed studies on the minimal structural requirements of the peptidoglycan ligands needed for NOD1 or NOD2 activation revealed that the MurNAc moiety is not required for NOD1 activation as the D-Glu-meso-DAP dipeptide (iE-DAP) is sufficient for detection and innate immune activation by this PRM (3,9). NOD2, on the other hand, can only be activated by muramyl dipeptides that have an intact MurNAc ring structure, and the sugar has to be attached to a dipeptide moiety (L-Ala-D-Glu or L-Ala-D-isoGln) (3,10). Importantly, studies have shown that both NOD1 and NOD2 can directly bind to TriDAP and MDP, respectively, thus showing that NOD1 and NOD2 are bona fide cytoplasmic receptors (11-13), and that this interaction requires the leucine-rich repeat region of NOD1 and NOD2 proteins (14,15).

While functional and binding studies demonstrate that NOD proteins have an extreme specificity for certain peptidoglycan fragments that is conserved in multiple vertebrates, recent studies have suggested that NOD signaling could also be triggered by viral infection (16), small Rho GTPases regulating cytoskeleton remodeling (17), and endoplasmic reticulum (ER) stress (18), implying that peptidoglycan-independent mechanisms of NOD stimulation may exist. This suggests that in addition to their high specificity towards peptidoglycan fragments, NOD1 and NOD2 may serve as promiscuous sensors of multiple and unrelated cellular stresses. Here, we aimed to better characterize how NOD proteins trigger
pro-inflammatory signaling in response to ER stress. While we confirm that thapsigargin, a specific inhibitor of the ER sarcoplasmic or endoplasmic reticulum calcium ATPase family (SERCA) calcium pump, triggers pro-inflammatory signaling in a NOD-dependent manner (18), our results suggest that this effect is actually mediated by the Ca\textsuperscript{2+}-dependent internalization of peptidoglycan trace fragments found in the fetal calf serum added to cell culture media. Of note, low levels of peptidoglycan in human serum have already been reported, and were shown to stimulate hyphal growth of *Candida albicans* (19). Together, our observations suggest that cellular perturbations that lead to increased intracellular Ca\textsuperscript{2+} levels may inadvertently trigger NOD-dependent signalling through the internalization of peptidoglycan contaminants, which offers an alternative explanation for the proposed promiscuous activation of NOD receptors by multiple unrelated stresses. These observations also suggest that chronic homeostatic peptidoglycan sensing by NOD proteins may impact multiple cellular processes in ways that have been overlooked previously and open up interesting questions in innate immunity, relating to understanding the physiological role of circulating peptidoglycan at homeostasis, both at the cellular and the tissue level.

**Results and Discussion**

The human intestinal epithelial cell line HCT116, either wild type (WT) or knockout out through CRISPR-Cas9 for *NOD1*, *NOD2* or both *NOD1* and *NOD2* (*NOD1/2* double knockout or DKO) described previously (20) were stimulated with the ER stress inducer thapsigargin, which inhibits the SERCA pump that transports Ca\textsuperscript{2+} to the ER lumen. While thapsigargin induced ER stress similarly in the four cell lines tested, as determined by the transcriptional upregulation of the *HSPA5* gene (that encodes heat shock protein BiP/GRP78) (Fig. 1A), transcriptional upregulation of the pro-inflammatory cytokines *CXCL1* (Fig. 1A), *IL-8* (Fig. S1A) as well as the chemokine *CCL20* (Fig. S1A) was significantly blunted in *NOD2* KO and *NOD1/2* DKO HCT116 cells, in line with previous results (18). In *NOD1* KO cells, a non-significant trend for reduced expression of *CXCL1* and *IL-8* was also noticed following thapsigargin stimulation, and significant reduction of *CCL20* expression...
was observed (Figs. 1A and S1A). Together, these results suggest that, in HCT116 cells, NOD2 and to a lesser extent NOD1, contribute to the upregulation of pro-inflammatory signaling induced by thapsigargin. On average, NOD1/2 DKO cells displayed an 8.35 fold reduction in CXCL1 induction following thapsigargin stimulation, a 6.33 fold reduction in IL-8 expression, and a 2.85 fold reduction in CCL20 expression.

To demonstrate that this effect requires the NOD adaptor protein RIP2, we engineered HCT116 RIPK2 KO cells by targeting the RIPK2 gene (encoding RIP2) using CRISPR-Cas9. As expected, RIPK2-targeted cells were insensitive to the synthetic NOD ligands MDP and iE-DAP in an NF-κB luciferase assay (Fig. S2A). Similar to NOD1/2 DKO cells, RIPK2 KO HCT116 cells displayed normal induction of ER stress-dependent HSPA5 (Fig. 1B), but significantly reduced upregulation of CXCL1 (Fig. 1B), IL-8 and CCL20 (Fig. S1B). On average, RIPK2 KO cells displayed a 3.82 fold reduction in CXCL1 induction following thapsigargin stimulation, a 13.48 fold reduction in IL-8 expression, and a 3.04 fold reduction in CCL20 expression. To further validate our findings, we reproduced these results with other clones of NOD1/2 DKO and RIPK2 KO cells (Fig. S2B-C). Finally, we isolated primary intestinal organoids from WT and Nod1/2 DKO mice and stimulated those with thapsigargin. In previous work, we identified using RNAseq that thapsigargin potently stimulated cytokine expression in murine organoids (21). Similar to our results with the human intestinal cell line HCT116, we observed that while thapsigargin triggered comparable expression of Hspa5 (encoding BiP) in WT and Nod1/2 DKO organoids, Cxcl1 expression was significantly blunted in Nod1/2 DKO organoids (Fig. 1C), although the effect was not as drastic as in HCT116 cells, suggesting that NOD-dependent signalling only represents a subset of thapsigargin-dependent pro-inflammatory cascades in murine organoids. Together, these results confirm the previous findings (18) that NOD1 and NOD2 are critical for inflammatory signalling induced by thapsigargin.

We next aimed to investigate if other ER stress inducers trigger pro-inflammatory signalling in a NOD-dependent manner in HCT116 cells. We first used tunicamycin, a molecule that induces ER stress by preventing protein
glycosylation in the ER lumen, thereby provoking the accumulation of misfolded proteins in the ER. This mechanism of ER stress induction is distinct from the one triggered by thapsigargin, which relies on the inhibition of Ca\textsuperscript{2+} accumulation in the ER lumen. Interestingly, while tunicamycin induced potent upregulation of $HSPA5$ expression in WT, $NOD1$ KO, $NOD2$ KO and $NOD1/2$ DKO HCT116 cells (Fig. 2A), it did not stimulate expression of $CXCL1$ or $IL8$ (Fig. 2A), suggesting that induction of pro-inflammatory signalling by thapsigargin is likely caused by the effect of the inhibitor on intracellular Ca\textsuperscript{2+} levels rather than caused by ER stress per se. To confirm these findings, we induced ER stress by a third mechanism, namely the targeting and degradation of the BiP protein by the SubAB toxin of Shiga toxigenic $Escherichia$ $coli$ (STEC), which causes ER stress as a consequence of reduced levels of luminal BiP chaperone (22,23). SubAB strongly induced upregulation of $HSPA5$ expression in WT, $NOD1$ KO, $NOD2$ KO and $NOD1/2$ DKO HCT116 cells, while a mutant toxin unable to cleave BiP was unable to do so (Fig. 2B). However, SubAB did not trigger expression of $CXCL1$ or $IL8$, similar to tunicamycin (Fig. 2B). These results further demonstrated that at least in HCT116 cells, ER stress does not induce NF-\kappaB-dependent pro-inflammatory cytokines such as $CXCL1$ and $IL8$, and that thapsigargin action on pro-inflammatory signalling is likely caused by its capacity to rise intracellular Ca\textsuperscript{2+} concentration.

To validate the plausible role of intracellular Ca\textsuperscript{2+} levels in NOD-dependent pro-inflammatory signalling, WT and $NOD1/2$ DKO cells were stimulated with A23187, a Ca\textsuperscript{2+} ionophore that causes massive influx of Ca\textsuperscript{2+} into the cytosol. As expected, stimulation with A23187 induced upregulation of $HSPA5$ in both WT and $NOD1/2$ DKO cells (Fig. 3A). Similarly to thapsigargin, A23187 also stimulated expression of $CXCL1$ and $IL8$, which was significantly blunted in $NOD1/2$ DKO cells (Fig. 3A), thus showing that the rise intracellular Ca\textsuperscript{2+} levels caused NOD-dependent pro-inflammatory signalling. Additionally, using BAPTA-AM, a cell-permeant Ca\textsuperscript{2+} chelator, we directly tested the role of intracellular Ca\textsuperscript{2+} levels in thapsigargin-stimulated cells. While BAPTA-AM did not significantly affect ER stress induced by thapsigargin (Fig. 3B), in line with the
fact that BAPTA-AM does not prevent thapsigargin-dependent depletion of luminal ER stores of Ca\(^{2+}\) but neutralizes the effects of cytosolic Ca\(^{2+}\), it blunted the induction of CXCL1 and IL8 expression in WT cells but not in NOD1/2 DKO cells. As a result, thapsigargin-induced expression of pro-inflammatory cytokines was not significantly dependent on NOD1/2 in BAPTA-AM-treated cells, although a trend for decreased expression was still observed in NOD1/2 DKO cells (Fig. 3B), possibly because BAPTA-AM was unable to fully neutralize the effects of intracellular Ca\(^{2+}\). This suggests that the rise of intracellular Ca\(^{2+}\) levels caused NOD-dependent pro-inflammatory signalling in HCT116 cells.

A depletion in Ca\(^{2+}\) stores in the ER, associated with a rise in intracellular Ca\(^{2+}\) levels, acts as a signal for the activation of a family of plasma membrane calcium release-activated channels (CRACs), which triggers internalization of extracellular Ca\(^{2+}\) as a means to regenerate ER Ca\(^{2+}\) stores (24). Therefore, to determine whether extracellular Ca\(^{2+}\) was mobilized during thapsigargin-induced NOD-dependent cytokine expression, WT and NOD1/2 DKO HCT116 were stimulated with thapsigargin in the presence of a DMNP-EDTA, a cell-impermeant Ca\(^{2+}\) chelator. Similar to the results obtained with BAPTA-AM, DMNP-EDTA did not impact HSPA5 expression induced by thapsigargin, but significantly blunted CXCL1 (by 2.56 fold) and IL8 expression (by 2.16 fold) in WT cells without significantly affecting expression of these genes in NOD1/2 DKO cells (Fig. 3C). However, despite the effect of DMNP-EDTA, thapsigargin-induced expression of CXCL1 and IL8 remained significantly greater in WT as compared to NOD1/2 DKO cells (Fig. 3C). Finally, to explore the possible contribution of CRACs to the effects observed, the specific inhibitor GSK-7975A was used, which showed results similar to those obtained with DMNP-EDTA (Fig. S3). Taken together, we conclude that internalization of extracellular Ca\(^{2+}\) through CRAC channels contributes yet does not account for the whole NOD-dependent cytokine response to thapsigargin stimulation.

We next aimed to delineate the mechanism by which an increase in intracellular Ca\(^{2+}\) levels causes NOD-dependent pro-inflammatory signalling. We reasoned that since Ca\(^{2+}\) regulates vesicular trafficking, exocytosis and
recycling of lysosomes (25,26), it could thus in turn impact on the dynamic rate at which cells perform endocytosis. In addition, Ca\(^{2+}\) internalization through CRACs also contributes to endocytosis (27,28). This led us to speculate that the Ca\(^{2+}\)-dependent signal that triggers NOD-dependent activation in thapsigargin-stimulated cells could be coming from a factor within the extracellular milieu and be brought in by endocytosis. To test this hypothesis, WT and NOD1/2 DKO cells were grown in synthetic Hanks' Balanced Salt Solution (HBSS) medium supplemented with Ca\(^{2+}\), in the presence or absence of 10% fetal calf serum (FCS) and were then stimulated with thapsigargin. Interestingly, NOD-dependent induction of IL8 following thapsigargin stimulation was only observed in the presence of serum, as thapsigargin was unable to trigger IL8 expression when cells were passaged in a medium containing only HBSS with Ca\(^{2+}\) prior to thapsigargin stimulation (Fig. 4A). This effect was Ca\(^{2+}\)-dependent, since it was lost when serum was added to cells grown in HBSS without Ca\(^{2+}\) (Fig. S4A), again arguing for the role played by extracellular Ca\(^{2+}\) in these responses. Importantly, the absence of serum did not make the cells refractory to NOD-dependent cytokine expression nonspecifically, since addition of the NOD2 ligand L18-MDP to cells incubated in HBSS with Ca\(^{2+}\) could still induce expression of IL8 and CXCL1 (Fig. S4B). Finally, we boiled the FCS and filtered it to remove molecules with a molecular weight > 3kDa. This boiled/filtered FCS was still able to potentiate NOD-dependent stimulation of IL8 following stimulation (Fig. S3C). This suggests that a heat-resistant small molecule present in FCS drives Ca\(^{2+}\)-dependent stimulation of NOD1/2 proteins by thapsigargin.

Since internalization of extracellular peptidoglycan into epithelial cells and subsequent activation of NOD1 and NOD2 occurs by clathrin- and dynamin-dependent endocytosis (29,30), we next aimed to define if the active molecule(s) in FCS needed to be internalized by endocytosis to drive thapsigargin-mediated stimulation of NOD proteins. To do so, WT and NOD1/2 DKO HCT116 cells were grown in a normal medium supplemented with 10% FCS and stimulated with thapsigargin in the presence or absence of dynasore, which specifically inhibits endocytosis. We observed that dynasore treatment
potently decreased \( IL8 \) expression induced by thapsigargin and abolished the NOD dependency of this stimulation (Fig. 4B), implying that the effect required endocytosis. We concluded that a heat-resistant small molecule present in FCS is brought in by endocytosis to stimulate NOD1/2 proteins during thapsigargin stimulation.

All the experiments above point to likely presence of peptidoglycan contaminants in the cell culture grade FCS in which our cells were grown, and that these contaminants would be internalized by endocytosis at a higher rate following an increase in intracellular \( \text{Ca}^{2+} \), resulting in NOD-dependent stimulation of pro-inflammatory signalling. A previous study similarly reported that peptidoglycan traces found in human serum were responsible for hyphal growth of \textit{Candida albicans} (19). Moreover, while this manuscript was under evaluation, a new study demonstrated the presence of MDP and other peptidoglycan fragments in the blood of multiple warm-blooded animals (31). To directly determine if our cell culture FCS contained peptidoglycan fragments, high-pressure liquid chromatography coupled to mass spectrometry was conducted on two separate batches of FCS, which revealed the presence of multiple muramyl peptides and peptidoglycan derived peptides (Fig. S5). In particular, several muramyl peptides identified, including GlcNAc-MurNAc-L-Ala-D-Gln-\textit{meso}DAP (GM-TriDAP), GlcNAc-MurNAc-L-Ala-D-Gln (GMDP) and L-Ala-D-Gln-\textit{meso}DAP (TriDAP) (Fig. 4C), are known activators of NOD1/2 (3). Similar results were obtained when sera from laboratory mice housed in our facility were tested (\textit{data not shown}), suggesting that traces of circulating peptidoglycan in serum may be a common feature.

There are several direct implications of our observations: first, our results provide an alternative explanation for the previous implication of NOD proteins as mediators of inflammatory signalling in response to ER stress (18), by showing that an increase in intracellular \( \text{Ca}^{2+} \) levels, instead of ER stress itself, caused internalization of trace contaminants of peptidoglycan from the cell culture serum; second, our results suggest that caution should be taken when analyzing if a given stimulus induces NF-\( \kappa \text{B} \)-dependent pro-inflammatory signalling in a NOD-dependent manner, since multiple pathways can cause
transient increase in intracellular Ca\textsuperscript{2+} levels, which could in turn trigger internalization of peptidoglycan contaminants. In particular, it was shown that perturbation of the actin cytoskeleton through Rho GTPases could modulate NF-κB-dependent pro-inflammatory signalling in a NOD-dependent manner (17). To begin exploring the aforementioned hypothesis, WT and NOD1/2 DKO cells cultured in serum supplemented HBSS were stimulated with Cytochalasin D (CytD), which disrupts the actin cytoskeleton polymerization, in the presence or absence of extracellular Ca\textsuperscript{2+}. Interestingly, we observed that stimulation with CytD in the presence Ca\textsuperscript{2+} induced expression of IL-8 and CXCL1 (albeit more modestly than thapsigargin did) in a NOD-dependent and calcium-dependent manner, while HSPA5 expression was not induced (Fig. S6). This suggests that multiple cellular perturbations could trigger NOD-dependent induction of pro-inflammatory pathways if they trigger mobilization of Ca\textsuperscript{2+} towards the intracellular compartment.

More generally, the presence of trace elements of peptidoglycan in animal serum may be of fundamental importance for physiology, although its implication has not yet been carefully evaluated. Interestingly, early studies have demonstrated that circulating peptidoglycan-derived muramyl peptides were able to regulate slow-wave sleep in rabbits (32-34). More recently, it was shown that translocation of peptidoglycan fragments from the intestinal microbiota into the circulation induced functional priming of neutrophils in a NOD1-dependent manner in mice (35), suggesting that these peptidoglycan fragments were sufficient to enhance systemic innate immunity. Since systemic administration of muramyl peptides has been shown to have numerous physiological consequences, from boosting innate and adaptive immune responses (36-38) to triggering protection against obesity-induced insulin tolerance (39) in the case of NOD2 agonists (and opposite effects in the case of NOD1 activators [39,40]), it is tempting to speculate that tonic low-level activation of NOD1/2 systemically caused by circulating peptidoglycan fragments may have far-reaching implications for host physiology that need to be fully characterized.
Experimental Procedures

Cell culture and reagents

The human epithelial HCT116 cell line (American Type Culture Collection) was cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50 IU penicillin, and 50µg/ml streptomycin (Wisent Bio Products). Cells were maintained in 95% air, 5% CO₂ at 37°C. Endotoxin-free FCS and phosphate-buffered saline (PBS) were from Wisent (Saint-Bruno-de-Montarville, Quebec, Canada). In some experiments, cells were washed with PBS and cell culture medium was replaced with Hank’s Balanced Salt Solution (Thermo Fisher) supplemented or not with CaCl₂ (Sigma; 140µg/ml). In various experiments, cells were treated with 10µg/ml of MDP, 10µg/ml of iEDAP, 200ng/ml L-18 MDP obtained from Invivogen; 0.1µg/ml of Thapsigargin, 1µg/ml of Tunicamycin, 1µM of A23187, 80µM of Dynasore monohydrate, 15µM of BAPTA-AM, 10µM of CRAC inhibitor (GSK7975A), 5µM of Cytochalasin D obtained from Sigma; and 200µM of DMNP-EDTA (cell impermeant) was purchased from Invitrogen, ThermoFisher Scientific. SubAB and SubA272B, a kind gift from Drs James and Adrienne Paton (University of Adelaide, Australia) were used at a concentration of 50ng/ml.

Primary murine organoids

To generate organoid cultures, crypts from the small intestines of mice were extracted as previously described (41). Briefly, the villi of the small intestine were removed by scraping, followed by washing with cold PBS. The remaining tissue were then homogenized and incubated in 2mM EDTA in PBS for 30min at 4°C, followed by vigorous washing in PBS several times to obtain crypt-enriched supernatant fractions. The supernatant fractions were then passed through a cell strainer, pelleted at 300g for 5min at 4°C and resuspended in 50µl Matrigel (Corning). The crypt-containing organoids were cultured by plating onto the center of a 24-well plate and grown in 500µl crypt culture medium supplemented with growth factors (R-spondin 1, Noggin, EGF). Organoids were allowed to grow 7 days, followed by passaging onto 6-well plates for stimulation (roughly 10-15 isolated organoids).
RNA isolation and quantitative RT-PCR

RNA samples were prepared using the GeneJET™ RNA Purification Kit (Thermo Scientific) according to the manufacture’s protocol. Eluted RNA was treated with DNase I (Fermentas) at 37°C for 1h to remove genomic DNA. cDNA was prepared from 1μg of total RNA using Oligo(dT), random hexamers, dNTPs, RNase OUT (Invitrogen) and M-MLV Reverse Transcriptase (Sigma). cDNA was diluted accordingly and prepared in 12μL reactions using PowerUp SYBR® Green Mastermix (Applied Biosystems). The CFX384 Touch™ Real-Time PCR Detection System (BioRad) was used to obtain the raw Ct values. Results were either analyzed using the $2^{-\Delta CT}$ formula normalizing target gene expression to the TBP housekeeping control or fold change was calculated by $2^{-\Delta\Delta CT}$ formula.

NF-κB luciferase assay

To measure NF-κB luciferase activity, 250,000 HCT116 cells (WT or RIPK2 KO) were plated per well and transfected with beta-galactosidase, NF-κB luciferase reporter and pcDNA3 plasmids for 24h. 10μg of MDP or iE-DAP were added directly to the cell culture medium at the time of transfection, as previously (3). Cells were then gently washed with PBS and lysed in luciferase buffer, followed by incubation at room temperature for 10min. 10μl of each cell lysate was then added to a black 96-well plate along with 100μl of luciferin buffer and luminescence was read using the Victor3 plate reader. To measure expression of the beta-galactosidase construct (transfection control) for normalization, 10 μl of original cell lysates were added to 100μl of the an ONPG buffer, incubated at 37°C for 30min and luminescence was read. The beta-galactosidase values were then used to normalize the absorbance values obtained for each sample.

LC/MS analysis of serum samples

Aliquots of FCS from different lots were tested as well as serum from laboratory mice kept in our facility in specific pathogen free (SPF) conditions. 20 μl of sera were loaded into the LC-MS platform. The LC-MS platform consisted of an Ultimate 3000 UHPLC coupled to a Q-Exactive mass spectrometer equipped with a HESI II source (Thermo Scientific). Control of the system was performed using Thermo XCalibur 2.2 software and
Chromeleon 7.2 software, with data processing conducted using Thermo Scientific Quan Browser. Separation by liquid chromatography was conducted on a Thermo Scientific Hypersil Gold C18 column (50mm × 2.1 mm, 1.9µm particle size). The pump was run at a flow rate of 300 µl/min. Solvent A was water containing 0.1% formic acid; solvent B was acetonitrile containing 0.1% formic acid. The gradient was: 0min, 5% B; 1min, 5% B; 2min, 30% B; 3min, 30% B; 4min, 50% B; 7min, 80% B; 9min, 80% B; 10min, 98% B; 11min 98% B; 12min, 5% B; 18min, 5% B. Autosampler temperature was maintained at 10°C and injection volume was 20µl. Data collection was done in positive ionization mode with MS1 scan range m/z 350–1200, resolution 70,000, AGC target of 3e6 and a maximum injection time of 100 ms, MS2 data was collected using a TOP5 method, 0.4 m/z isolation window, 30 NCE, 17,500 resolution, AGC target 1e5 and a maximum injection time of 50 ms. Data collected were analyzed using Quan Browser.

**Statistical analysis**

Significant differences between mean values were evaluated using a two-way ANOVA with multiple comparisons using Prism (GraphPad Inc). In all qRT-PCR experiments presented in this study, each point represents the average (from two or three technical replicates) from one experiment. Data from at least three independent experiments were pooled to generate each of the graphs presented. (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05).

**qRT-PCR primers**

- mouse RPL19 (Forward): 5’-GCATCCTCATGGAGCACAAT-3’
- mouse RPL19 (Reverse): 5’-CTGGTCAGCCAGGAGCTT-3’
- mouse Cxcl1 (Forward): 5’-AGACCATGGCTGGGATTCAC-3’
- mouse Cxcl1 (Reverse): 5’-AGTGTGGCTATGACTTCGGT-3’
- mouse Hspa5 (Forward): 5’-GCCTCATCGGACGACTT-3’
- mouse Hspa5 (Reverse): 5’-GGGGCAAATGTCTTGGTT-3’
- human TBP (Forward): 5’-GGGCATTATTTGTGCACTGAGA-3’
- human TBP (Reverse): 5’-TAGCAGCACGGTATGAGCAACT-3’
- human IL-8 (Forward): 5’-CCACCGGAAGGAACCATCTC-3’
human IL-8 (Reverse): 5’-TTCCTTGGGGTCCAGACAGA-3’
human CXCL1 (Forward): 5’-CACACTCAAGAATGGGCGGA-3’
human CXCL1 (Reverse): 5’-ACTATGGGGGATGCAGGATTG-3’
human HSPA5 (Forward): 5’-GAACGTCTGATTGGCGATGC-3’,
human HSPA5 (Reverse): 5’-TCAACCACCTTGAAACGGCAA-3’

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Conflict of Interest declaration
The authors declare that they have no conflicts of interest with the contents of this article.

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References

1. Philpott, D. J., Sorbara, M. T., Robertson, S. J., Croitoru, K., and Girardin, S. E. (2014) NOD proteins: regulators of inflammation in health and disease. *Nature reviews. Immunology* 14, 9-23

2. Mukherjee, T., Hovingh, E. S., Foerster, E. G., Abdel-Nour, M., Philpott, D. J., and Girardin, S. E. (2018) NOD1 and NOD2 in inflammation, immunity and disease. *Archives of biochemistry and biophysics*

3. Girardin, S. E., Travassos, L. H., Herve, M., Blanot, D., Boneca, I. G., Philpott, D. J., Sansonetti, P. J., and Mengin-Lecreux, D. (2003) Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *The Journal of biological chemistry* 278, 41702-41708

4. Girardin, S. E., Boneca, I. G., Carneiro, L. A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M. K., Labigne, A., Zahringer, U., Coyle, A. J., DiStefano, P. S., Bertin, J., Sansonetti, P. J., and Philpott, D. J. (2003) Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584-1587

5. Chamaillard, M., Hashimoto, M., Horie, Y., Masumoto, J., Qiu, S., Saab, L., Ogura, Y., Kawasaki, A., Fukase, K., Kusumoto, S., Valvano, M. A., Foster, S. J., Mak, T. W., Nunez, G., and Inohara, N. (2003) An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nature immunology* 4, 702-707

6. Girardin, S. E., Tournebize, R., Mavris, M., Page, A. L., Li, X., Stark, G. R., Bertin, J., DiStefano, P. S., Yaniv, M., Sansonetti, P. J., and Philpott, D. J. (2001) CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO reports* 2, 736-742

7. Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., Foster, S. J., Moran, A. P., Fernandez-Luna, J. L., and Nunez, G. (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *The Journal of biological chemistry* 278, 5509-5512

8. Girardin, S. E., Boneca, I. G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D. J., and Sansonetti, P. J. (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *The Journal of biological chemistry* 278, 8869-8872
9. Magalhaes, J. G., Philpott, D. J., Nahori, M. A., Jehanno, M., Fritz, J., Le Bourhis, L., Viala, J., Hugot, J. P., Giovannini, M., Bertin, J., Lepoivre, M., Mengin-Lecreulx, D., Sansonetti, P. J., and Girardin, S. E. (2005) Murine Nod1 but not its human orthologue mediates innate immune detection of tracheal cytotoxin. *EMBO reports* 6, 1201-1207

10. Rubino, S. J., Magalhaes, J. G., Philpott, D., Bahr, G. M., Blanot, D., and Girardin, S. E. (2013) Identification of a synthetic muramyl peptide derivative with enhanced Nod2 stimulatory capacity. *Innate immunity* 19, 493-503

11. Grimes, C. L., Ariyananda Lde, Z., Melnyk, J. E., and O’Shea, E. K. (2012) The innate immune protein Nod2 binds directly to MDP, a bacterial cell wall fragment. *Journal of the American Chemical Society* 134, 13535-13537

12. Laroui, H., Yan, Y., Narui, Y., Ingersoll, S. A., Ayyadurai, S., Charania, M. A., Zhou, F., Wang, B., Salaita, K., Sitaraman, S. V., and Merlin, D. (2011) L-Ala-gamma-D-Glu-meso-diaminopimelic acid (DAP) interacts directly with leucine-rich region domain of nucleotide-binding oligomerization domain 1, increasing phosphorylation activity of receptor-interacting serine/threonine-protein kinase 2 and its interaction with nucleotide-binding oligomerization domain 1. *The Journal of biological chemistry* 286, 31003-31013

13. Mo, J., Boyle, J. P., Howard, C. B., Monie, T. P., Davis, B. K., and Duncan, J. A. (2012) Pathogen sensing by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is mediated by direct binding to muramyl dipeptide and ATP. *The Journal of biological chemistry* 287, 23057-23067

14. Girardin, S. E., Jehanno, M., Mengin-Lecreulx, D., Sansonetti, P. J., Alzari, P. M., and Philpott, D. J. (2005) Identification of the critical residues involved in peptidoglycan detection by Nod1. *The Journal of biological chemistry* 280, 38648-38656

15. Tanabe, T., Chamaillard, M., Ogura, Y., Zhu, L., Qiu, S., Masumoto, J., Ghosh, P., Moran, A., Predergast, M. M., Tromp, G., Williams, C. J., Inohara, N., and Nunez, G. (2004) Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. *The EMBO journal* 23, 1587-1597

16. Lupfer, C., Thomas, P. G., Anand, P. K., Vogel, P., Milasta, S., Martinez, J., Huang, G., Green, M., Kundu, M., Chi, H., Xavier, R. J., Green, D. R., Lamkanfi, M., Dinarello, C. A., Doherty, P. C., and Kanneganti, T. D. (2013) Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection. *Nature immunology* 14, 480-488

17. Keestra, A. M., Winter, M. G., Auburger, J. J., Frassle, S. P., Xavier, M. N., Winter, S. E., Kim, A., Poon, V., Ravesloot, M. M., Waldenmaier, J. F., Tsolis, R. M.,
Eigenheer, R. A., and Baumler, A. J. (2013) Manipulation of small Rho GTPases is a pathogen-induced process detected by NOD1. *Nature* **496**, 233-237

18. Keestra-Gounder, A. M., Byndloss, M. X., Seyffert, N., Young, B. M., Chavez-Arroyo, A., Tsai, A. Y., Cevallos, S. A., Winter, M. G., Pham, O. H., Tiffany, C. R., de Jong, M. F., Kerrinnes, T., Ravindran, R., Luciw, P. A., McSorley, S. J., Baumler, A. J., and Tsolis, R. M. (2016) NOD1 and NOD2 signalling links ER stress with inflammation. *Nature* **532**, 394-397

19. Xu, X. L., Lee, R. T., Fang, H. M., Wang, Y. M., Li, R., Zou, H., Zhu, Y., and Wang, Y. (2008) Bacterial peptidoglycan triggers Candida albicans hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell host & microbe* **4**, 28-39

20. Gaudet, R. G., Guo, C. X., Molinaro, R., Kottwitz, H., Rohde, J. R., Dangeard, A. S., Arrieumerlou, C., Girardin, S. E., and Gray-Owen, S. D. (2017) Innate Recognition of Intracellular Bacterial Growth Is Driven by the TIFA-Dependent Cytosolic Surveillance Pathway. *Cell reports* **19**, 1418-1430

21. Tsalikis, J., Pan, Q., Tattoli, I., Maisonneuve, C., Blencowe, B. J., Philpott, D. J., and Girardin, S. E. (2016) The transcriptional and splicing landscape of intestinal organoids undergoing nutrient starvation or endoplasmic reticulum stress. *BMC genomics* **17**, 680

22. Chong, D. C., Paton, J. C., Thorpe, C. M., and Paton, A. W. (2008) Clathrin-dependent trafficking of subtilase cytotoxin, a novel AB5 toxin that targets the endoplasmic reticulum chaperone BiP. *Cellular microbiology* **10**, 795-806

23. Wolfson, J. J., May, K. L., Thorpe, C. M., Jandhyala, D. M., Paton, J. C., and Paton, A. W. (2008) Subtilase cytotoxin activates PERK, IRE1 and ATF6 endoplasmic reticulum stress-signalling pathways. *Cellular microbiology* **10**, 1775-1786

24. Yeung, P. S., Yamashita, M., and Prakriya, M. (2017) Pore opening mechanism of CRAC channels. *Cell calcium* **63**, 14-19

25. Di Paola, S., Scotto-Rosato, A., and Medina, D. L. (2018) TRPML1: The Ca\textsuperscript{2+}retaker of the lysosome. *Cell calcium* **69**, 112-121

26. Ganley, I. G., Wong, P. M., and Jiang, X. (2011) Thapsigargin distinguishes membrane fusion in the late stages of endocytosis and autophagy. *Autophagy* **7**, 1397-1399

27. Voronina, S., Collier, D., Chvanov, M., Middlehurst, B., Beckett, A. J., Prior, I. A., Criddle, D. N., Begg, M., Mikoshiba, K., Sutton, R., and Tepikin, A. V. (2015) The role of Ca\textsuperscript{2+} influx in endocytic vacuole formation in pancreatic acinar cells. *The Biochemical Journal* **465**, 405-412
28. Zeng, B., Chen, G. L., Garcia-Vaz, E., Bhandari, S., Daskoulidou, N., Berglund, L. M., Jiang, H., Hallett, T., Zhou, L. P., Huang, L., Xu, Z. H., Nair, V., Nelson, R. G., Ju, W., Kretzler, M., Atkin, S. L., Gomez, M. F., and Xu, S. Z. (2017) ORAI channels are critical for receptor-mediated endocytosis of albumin. *Nature communications* **8**, 1920

29. 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. *The journal of biological chemistry* **284**, 23818-23829

30. Marina-Garcia, N., Franchi, L., Kim, Y. G., Hu, Y., Smith, D. E., Boons, G. J., and Nunez, G. (2009) Clathrin- and dynamin-dependent endocytic pathway regulates muramyl dipeptide internalization and NOD2 activation. *J Immunol* **182**, 4321-4327

31. Huang, Z., Wang, J., Xu, X., Wang, H., Qiao, Y., Chu, W. C., Xu, S., Chai, L., Cottier, F., Pavelka, N., Oostjen, M., Joosten, L. A. B., Netea, M., Ng, C. Y. L., Leong, K. P., Kundra, P., Lam, K. P., Pettersson, S., and Wang, Y. (2019) Antibody neutralization of microbiota-derived circulating peptidoglycan dampens inflammation and ameliorates autoimmunity. *Nature microbiology*

32. Krueger, J. M., Karnovsky, M. L., Martin, S. A., Pappenheimer, J. R., Walter, J., and Biemann, K. (1984) Somnogenic and pyrogenic activities of some naturally occurring muramyl peptides; correlations with mass spectrometric structure determination. *The Journal of biological chemistry* **259**, 12659-12662

33. Krueger, J. M., Pappenheimer, J. R., and Karnovsky, M. L. (1982) Sleep-promoting effects of muramyl peptides. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 6102-6106

34. Martin, S. A., Karnovsky, M. L., Krueger, J. M., Pappenheimer, J. R., and Biemann, K. (1984) Peptidoglycans as promoters of slow-wave sleep. I. Structure of the sleep-promoting factor isolated from human urine. *The Journal of biological chemistry* **259**, 12652-12658

35. Clarke, T. B., Davis, K. M., Lysenko, E. S., Zhou, A. Y., Yu, Y., and Weiser, J. N. (2010) Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nature medicine* **16**, 228-231

36. Fraser-Smith, E. B., and Matthews, T. R. (1981) Protective effect of muramyl dipeptide analogs against infections of *Pseudomonas aeruginosa* or *Candida albicans* in mice. *Infection and immunity* **34**, 676-683
37. Leclerc, C., Juy, D., Bourgeois, E., and Chedid, L. (1979) In vivo regulation of humoral and cellular immune responses of mice by a synthetic adjuvant, N-acetyl-muramyl-L-alanyl-D-isoglutamine, muramyl dipeptide for MDP. *Cellular immunology* 45, 199-206

38. Lowy, I., Leclerc, C., Bourgeois, E., and Chedid, L. (1980) Inhibition of mitogen-induced polyclonal activation by by a synthetic adjuvant, muramyl dipeptide (MDP). *J Immunol* 124, 320-325

39. Cavallari, J. F., Fullerton, M. D., Duggan, B. M., Foley, K. P., Denou, E., Smith, B. K., Desjardins, E. M., Henriksbo, B. D., Kim, K. J., Tuinema, B. R., Stearns, J. C., Prescott, D., Rosenstiel, P., Coombes, B. K., Steinberg, G. R., and Schertzer, J. D. (2017) Muramyl Dipeptide-Based Postbiotics Mitigate Obesity-Induced Insulin Resistance via IRF4. *Cell metabolism* 25, 1063-1074 e1063

40. Chan, K. L., Tam, T. H., Boroumand, P., Prescott, D., Costford, S. R., Escalante, N. K., Fine, N., Tu, Y., Robertson, S. J., Prabaharan, D., Liu, Z., Bilan, P. J., Salter, M. W., Glogauer, M., Girardin, S. E., Philpott, D. J., and Klip, A. (2017) Circulating NOD1 Activators and Hematopoietic NOD1 Contribute to Metabolic Inflammation and Insulin Resistance. *Cell reports* 18, 2415-2426

41. Tattoli, I., Killackey, S. A., Foerster, E. G., Molinaro, R., Maisonneuve, C., Rahman, M. A., Winer, S., Winer, D. A., Streutker, C. J., Philpott, D. J., and Girardin, S. E. (2016) NLRX1 Acts as an Epithelial-Intrinsic Tumor Suppressor through the Modulation of TNF-Mediated Proliferation. *Cell reports* 14, 2576-2586
Figure Legends

Figure 1. Thapsigargin induces pro-inflammatory cytokine expression in a NOD-dependent manner

(A-B) Expression of HSPA5 and CXCL1 in wild type (WT), NOD1 knockout (KO), NOD2 KO and NOD1/2 double KO (DKO) HCT116 cells (A) or WT and RIPK2 KO HCT116 cells (B) following stimulation with 0.1µg/ml thapsigargin for 4 hours measured by qPCR. (C) Expression of Hspa5 and Cxcl1 in primary intestinal organoids from wild type (WT), and NOD1/2 double KO (DKO) mice following stimulation with 1µg/ml thapsigargin for 4 hours. Gene expression analysis was carried out by qRT-PCR. In all data sets, each point is from an independent experiment and is the average of three technical replicates. *, ** and **** represents p < 0.05, p < 0.01 and p < 0.0001, respectively. ns - not significant.

Figure 2. ER stress inducers that do not directly target Ca2+ stores do not trigger NOD-dependent pro-inflammatory cytokine expression

(A-B) Expression of HSPA5, CXCL1 and IL8 in wild type (WT), NOD1 knockout (KO), NOD2 KO and NOD1/2 double KO (DKO) HCT116 cells following stimulation with 1µg/ml tunicamycin (A) or 50ng/ml SubAB toxin from Shiga toxigenic Escherichia coli, either wild type (sAB) or mutated on position 272 (sA272B), which abolishes its activity, for 4 hours (B) and measured by qRT-PCR. In all data sets, each point is from an independent experiment and is the average of three technical replicates. ** and **** represents p < 0.01 and p < 0.0001, respectively. ns - not significant.

Figure 3. An increase in intracellular Ca2+ levels induces NOD-dependent pro-inflammatory signalling

(A) Expression of HSPA5, CXCL1 and IL8 in wild type (WT) and NOD1/2 double KO (DKO) HCT116 cells following stimulation with 1µg/ml A23187. (B-C) Expression of HSPA5, CXCL1 and IL8 in wild type (WT) and NOD1/2 double KO (DKO) HCT116
cells following treatment with 0.1µg/ml thapsigargin either in the presence or absence of 15µM BAPTA-AM, for 4 hours (B) or in the presence or absence of 200µM DMNP-EDTA for 1 hour (C). In all panels, gene expressions were measured by qRT-PCR and each point in all data sets is from an independent experiment and is the average of two or three technical replicates. ** and **** represents $p<0.01$ and $p<0.0001$, respectively. ns - not significant.

**Figure 4. Endocytosis of small molecules from cell culture serum, which contains peptidoglycan fragments, triggers NOD-dependent activation of pro-inflammatory signalling induced by thapsigargin**

(A) Expression of HSPA5 and IL8 in wild type (WT) and NOD1/2 double KO (DKO) HCT116 cells incubated overnight in Hanks' Balanced Salt Solution (HBSS) medium supplemented with 140µg/ml CaCl₂ in the presence or absence of 10% fecal calf serum (FCS) following stimulation with 0.1µg/ml thapsigargin for 4 hours. (B) Expression of HSPA5 and IL8 in WT and NOD1/2 DKO HCT116 cells following stimulation with 0.1µg/ml thapsigargin, in the presence or absence of 80 mM Dynasore, for 4 hours. Gene expression analysis was carried out by qRT-PCR. (C) LC-MS peak profile of 3 peptidoglycan fragments known to activate NOD1 and NOD2 (GM-TriDAP, GMDP and TriDAP) identified in FCS. In all data sets, each point is from an independent experiment and is the average of three technical replicates. ** and **** represents $p<0.01$ and $p<0.0001$, respectively. ns - not significant.
Figure 1

A

HSPA5

CXCL1

Relative mRNA Expression (log)

WT
NOD1 KO
NOD2 KO
NOD1/2 DKO

ns

B

HSPA5

CXCL1

Relative mRNA Expression (log)

WT
NOD1/2 DKO

ns

C

Hspa5

Cxcl1

Relative mRNA Expression

WT
NOD1/2 DKO

ns

Figure 1
**Figure 4**

(A) Relative mRNA expression of HSPA5 and IL8 in Ctl, Thap, Ctl Thap, HBSS+Ca²⁺, HBSS+Ca²⁺ + FCS conditions. Significant differences are indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(B) Relative mRNA expression of HSPA5 and IL8 in WT and NOD1/2 DKO conditions under HBSS+Ca²⁺ + FCS treatment. Significant differences are indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(C) Mass spectrometry analysis showing relative abundance of GM-TriDAP (m/z 2.33) and TriDAP (m/z 9.55) ions with retention time values of 7.97 and 9.55, respectively. Significant differences are indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Trace levels of peptidoglycan in serum underlie the NOD-dependent cytokine response to endoplasmic reticulum stress

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