NOD1 and NOD2 signalling links ER stress with inflammation

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Endoplasmic reticulum (ER) stress is a major contributor to inflammatory diseases, such as Crohn disease and type 2 diabetes1,2. ER stress induces the unfolded protein response, which involves activation of three transmembrane receptors, ATF6, PERK and IRE1α. Once activated, IRE1α recruits TRAF2 to the ER membrane to initiate inflammatory responses via the NF-κB pathway3. Inflammation is commonly triggered when pattern recognition receptors (PRRs), such as Toll-like receptors or nucleotide-binding oligomerization domain (NOD)-like receptors, detect tissue damage or microbial infection. However, it is not clear which PRRs have a major role in inducing inflammation during ER stress. Here we show that NOD1 and NOD2, two members of the NOD-like receptor family of PRRs, are important mediators of ER-stress-induced inflammation in mouse and human cells. The ER stress inducers thapsigargin and dithiothreitol trigger production of the pro-inflammatory cytokine IL-6 in a NOD1/2-dependent fashion. Inflammation and IL-6 production triggered by infection with Brucella abortus, which induces ER stress by infecting the type IV secretion system effector protein VceC into host cells4, is TRAF2, NOD1/2 and RIP2-dependent and can be reduced by treatment with the ER stress inhibitor tauroursodeoxycholate or an IRE1α kinase inhibitor. The association of NOD1 and NOD2 with pro-inflammatory responses induced by the IRE1α/TRAF2 signalling pathway provides a novel link between innate immunity and ER-stress-induced inflammation.

We investigated a potential role for NOD1 and NOD2 in the unfolded protein response (UPR), because this response triggers inflammation by activating IRE1α, a receptor that recruits TRAF2 to activate NF-κB (Extended Data Fig. 1a)5,6. NOD1 and NOD2 are two NLRs that contain major TRAF2 binding motifs7. Consistent with previous reports8,9, TRAF2 was required for NF-κB activation triggered by stimulation with canonical NOD1/2 ligands or mediated by auto-activation of NOD1, NOD2 or the NOD1/2 adaptor protein RIP2 (Extended Data Fig. 1b, c). To investigate whether NOD1 and NOD2 contribute to inflammatory responses during ER stress, we stimulated bone-marrow-derived macrophages (BMDMs) of wild-type (C57BL/6) mice or mice deficient for NOD1 and NOD2 (Nod1/2−/− mice) with the ER stress inducer thapsigargin, a specific inhibitor of the SERCA channel10. Thapsigargin treatment induced the UPR, as indicated by a NOD1/2-independent elevation in transcript levels of Hspa5 (encoding the ER-resident chaperone GRP78/BiP) and Chop (also known as Ddit3; Extended Data Fig. 2a, b), two genes controlled by the ER stress sensors ATF6 and PERK (Extended Data Fig. 1a). Thapsigargin treatment also significantly (P < 0.05) induced Il6 transcription (Fig. 1a) and IL-6 synthesis (Fig. 1b), while synthesis of TRAF2 and the chaperones SGT1 and HSP90 remained unchanged (Extended Data Fig. 2c). Both Il6 transcription and IL-6 synthesis were blunted in BMDMs from Nod1/2−/− mice compared to wild-type mice (Fig. 1a, b), which was not due to differences in cell death (Extended Data Fig. 2d). Similarly, IL-6 synthesis induced by the ER stress inducer dithiothreitol was abrogated in BMDMs from Nod1/2−/− mice (Extended Data Fig. 2e) which was not due to differences in cell death (Extended Data Fig. 2d). Thapsigargin-induced IL-6 synthesis could be blunted by treatment with the ER stress inhibitor TUDCA (Extended Data Fig. 2f). By contrast, stimulation of BMDMs with the canonical NOD2 ligand muramyl dipeptide (MDP) did not result in increased transcript levels of Hspa5 and Chop (Extended Data Fig. 2a, b), but led to increased synthesis of IL-6 (Extended Data Fig. 2e), which could not be inhibited by TUDCA treatment (Extended Data Fig. 2f). In conclusion, the TUDCA-resistant pathway of IL-6 production induced by stimulation with a canonical

Figure 1 | Thapsigargin induced IL-6 production is dependent on NOD1 and NOD2. a–c, BMDMs from Nod1/2−/− mice and wild-type littermates (n = 8) were stimulated with thapsigargin (a, b) and/or KIRA6 (c) and Il6 mRNA expression (a) and IL-6 protein synthesis (b, c) were measured. d, e, Nod1/2−/− mice and wild-type littermates (n = 7) were injected with thapsigargin, and IL-6 production in the serum (d) and Il6 mRNA expression in the spleen (e) were determined. Data are presented as mean ± s.e.m.
NOD2 ligand was distinct from the TUDCA-sensitive pathway of IL-6 production induced by thapsigargin (Extended Data Fig. 1a).

The IRE1α kinase domain is important for TRAF2 binding 4, while the IRE1α RNase domain functions in splicing a 26-nucleotide intronic sequence from the Xbp1 mRNA11. We thus investigated whether thapsigargin-induced IL-6 synthesis could be blunted by treatment with the IRE1α kinase inhibitor KIRA6 (1-[4-[8-amino-3-tert-butyldimazo[1,5-a]pyrazin-1-yl]napthalen-1-yl]-3-[3-[(tri-fluoromethyl)phenyl]urea]12, the IRE1α RNase inhibitor STF-083010 (N-[2-hydroxynaphthalen-1-yl]methylene]thiophene-2-sulfonamide)13 or the PERK inhibitor GSK2656157. Thapsigargin-induced IL-6 synthesis in BMDMs was blunted by treatment with the IRE1α kinase inhibitor KIRA6 (Fig. 1c) which was not due to differences in cell death (Extended Data Fig. 2d). The PERK inhibitor GSK2656157 and the IRE1α RNase inhibitor STF-083010 inhibited expression of Chop and Hspa5, respectively, but did not reduce thapsigargin-induced IL-6 synthesis (Extended Data Fig. 2g–j). Collectively, these data suggested that the IRE1α kinase branch of the ER stress response was most important for inducing IL-6 synthesis (Extended Data Fig. 1a).

To investigate the in vivo relevance of our observations, we injected mice with thapsigargin, which resulted in elevated levels of IL-6, KC (CXCL1) and MIP-1β (CCL4) in the serum (Fig. 1d, Extended Data Fig. 3a–c) and increased transcription of Il6 in the spleen, liver and kidney (P < 0.01) (Fig. 1e, Extended Data Fig. 3d, e) of wild-type mice, while induction of these pro-inflammatory responses was significantly blunted in Nod1/2−/− mice. Furthermore, thapsigargin-induced cytokine synthesis could be blunted by treatment with the ER stress inhibitor TUDCA (Extended Data Fig. 3a–c). Collectively, these data suggested that ER stress induced NOD1 and/or NOD2 signalling independently of peptidoglycan and that this pathway was only required for orchestrating the pro-inflammatory branch of the UPR, which leads to IL-6 production (Extended Data Fig. 1a).

ER stress has recently emerged as a response induced during infection with some bacterial pathogens (summarized in ref. 3). B. abortus is an ideal model organism to investigate induction of the UPR in vivo, because this pathogen has developed mechanisms to evade recognition by TLRs (reviewed in refs 15, 16). As a result, inflammatory responses induced during brucellosis are almost entirely dependent on a functional type IV secretion system 17, a virulence factor that injects effector proteins, including VceC, into the host cell cytosol 18. VceC translocates to the ER where it binds the ER chaperone BiP and induces an IRE1α-dependent induction of IL-6 production 5. Profiling of host responses elicited by interperitoneal infection of mice with B. abortus revealed elevated circulating levels of IL-6 (Extended Data Fig. 4a), IL-12p40 (Extended Data Fig. 4b), IFN-γ (Extended Data Fig. 4c), KC (Extended Data Fig. 4d), MIP-1β (Extended Data Fig. 4e), G-CSF (Extended Data Fig. 4f) and RANTES (Extended Data Fig. 4g). Blunting of these cytokine levels after treatment with TUDCA confirmed that inflammatory responses were triggered by ER stress (Extended Data Fig. 4). Intraperitoneal infection of mice with B. abortus induced elevated transcript levels of Il6, but this response was significantly (P < 0.05) blunted in mice infected with a B. abortus vceC mutant. The VceC-induced pro-inflammatory response was caused by ER stress, because elevated Il6 transcript levels during infection with the B. abortus wild type, but not those triggered by the vceC mutant, were significantly (P < 0.05) blunted after treatment with the ER stress inhibitor TUDCA (Fig. 2a), which was not due to altered bacterial numbers in the spleen (Extended Data Fig. 5a). Furthermore, Il6 transcript levels induced in the spleen and IL-6 synthesis induced in the serum of mice infected with the B. abortus wild type was significantly (P < 0.05) blunted by treatment with the IRE1α kinase inhibitor KIRA6 (Extended Data Fig. 5b and Fig. 2b), which was not due to altered bacterial numbers in the spleen (Extended Data Fig. 5c).

We next studied whether VceC-induced ER stress triggered NF-κB activation and IL-6 production through a pathway requiring NOD1 and/or NOD2 activity. B. abortus infection elicited significantly (P < 0.05) higher production of IL-6 (Fig. 2c) and a significant elevation of Il6 transcript levels (Extended Data Fig. 5d) in BMDMs from
wild-type mice compared to those from Nod1/2−/− mice. In contrast, infection with a vceC mutant elicited similar responses in BMDMs from wild-type and Nod1/2−/− mice. Differences in IL-6 synthesis were not caused by differences in bacterial numbers associated with BMDMs (Extended Data Fig. 5e). Similar results were obtained with BMDMs from Rip2−/− mice (Extended Data Fig. 5f). Ectopic expression of VceC in HEK293 cells induced NF-κB activation, which could be significantly blunted by transfecting cells with dominant negative forms of TRAF2 (P < 0.005) (Fig. 2d), NOD1 (P < 0.001), NOD2 (P < 0.001) or RIP2 (P < 0.001) (Fig. 2e). In contrast, transfection with a control protein (a dominant negative form of CDC42) did not reduce VceC-induced NF-κB activation (Fig. 2e). Collectively, these data suggest that VceC-triggered NF-κB activation requires NOD1, NOD2 and RIP2 activity.

To investigate the biological significance of NOD1/2-mediated responses to VceC, we infected Nod1/2−/− mice and wild-type littermates with the mutant. The B. abortus wild type elicited significantly higher levels of circulating IL-6 (Fig. 2f) and splenic Il6 gene transcription (Fig. 2g) in wild-type mice compared to Nod1/2−/− mice, although the latter animals did not exhibit reduced bacterial tissue load (Extended Data Fig. 5g). TUDCA treatment blunted IL-6 synthesis in wild-type mice but not in Nod1/2−/− mice (Fig. 2f). Responses triggered by the vceC mutant were not significantly different in wild-type and Nod1/2−/− mice (Fig. 2f, g). Similarly, the B. abortus wild type elicited significantly higher IL-6 synthesis (Fig. 2h) and splenic Il6 expression (Extended Data Fig. 5h) in wild-type mice compared to Rip2−/− mice, which was not due to differences in bacterial load (Extended Data Fig. 5i). These data supported the idea that the NOD1/NOD2/RIP2-signalling pathway was required for the VceC-dependent induction of pro-inflammatory responses during B. abortus infection in vivo.

B. abortus generally causes mild chronic inflammation during its persistence in the tissue of experimentally infected mice or within its natural bovine reservoir. However, in pregnant cows, the pathogen causes severe acute inflammation in the placenta, which leads to abortion. We next wanted to investigate the contribution of ER stress to acute placentalitis using the pregnant mouse model10. Bacterial numbers in the placenta of mice infected with B. abortus wild type at 5 days of pregnancy increased to 106 colony-forming units (CFU) per g tissue by day 13 after infection (corresponding to day 18 of gestation) (Extended Data Fig. 6a), which was accompanied by a significant (P < 0.05) increase in Il6 transcript levels in the placenta (Extended Data Fig. 6b) and acute placentalitis (Extended Data Figs 6c–e). This time point was chosen to investigate a potential role for VceC in causing placentalitis. Although the B. abortus wild type and the vceC mutant were recovered in similar numbers from the placenta (Extended Data Fig. 7a), the severity of placentalitis (Fig. 3a, b) and the Il6 transcript levels in the placenta (Fig. 3c) were significantly (P < 0.05) reduced in mice infected with the vceC mutant. Notably, while none of the pups survived an infection with the B. abortus wild type, in mice infected with the vceC mutant approximately 30% of the pups were viable at day 18 of gestation (Fig. 3d). These data suggest that VceC-induced inflammation contributes to both placentalitis and reduction of pup viability in this model.

Injecting B. abortus infected pregnant mice with the ER stress inhibitor TUDCA decreased Il6 transcript levels in the placenta (P < 0.001) (Fig. 3c), reduced the severity of placentalitis (P < 0.01) (Fig. 3a, b), and increased the viability of pups to approximately 40% (Fig. 3d), while recovery of bacteria from organs remained similar (Extended Data Fig. 7b). To examine whether the NOD1/2 signalling pathway contributed to placentalitis, pregnant Nod1/2−/− mice and wild-type littermate control mice were infected with B. abortus. Compared to infection of pregnant wild-type mice, B. abortus infection of pregnant Nod1/2−/− mice produced diminished transcript levels of Il6 in the placenta (P < 0.05) (Fig. 3e), reduced the severity of placentalitis (P < 0.005) (Fig. 3a, b), and increased the viability of pups to approximately 30% (Fig. 3d), while recovery of bacteria from organs remained unchanged.
To investigate whether the proposed pathway was induced during infections with other pathogens, we infected HEa cells with Chlamydia muridarum, a known ER stress inducer. Infection of HEa cells with C. muridarum or treatment with thapsigargin induced Il6 mRNA expression, which could be blunted by treatment with the IRE1α kinase inhibitor KIRA6 (Extended Data Fig. 8). Consistent with our model (Extended Data Fig. 1a), KIRA6 treatment did not blunt Il6 expression induced by stimulation of HEa cells with the NOD2 ligand MDP. Inhibition of NOD1 and NOD2 signalling by transfecting HEa cells with a dominant negative form of RIP2 blunted Il6 expression induced by C. muridarum infection, thapsigargin treatment or stimulation with MDP (Extended Data Fig. 8). These results suggest that the NOD1/ NOD2/RIP2 signalling pathway also detects ER stress generated during Chlamydia infection.

NOD1 and NOD2 are traditionally viewed as cytosolic sensors of bacterial peptidoglycan fragments. A clue that NOD2 may perform functions in addition to sensing canonical bacterial peptidoglycan ligands comes from the finding that this PRR activates innate immune responses during infection with influenza virus, a pathogen that triggers ER stress in infected host cells. Our results suggest that proinflammatory responses induced by ER stress are mediated through a NOD1/2-dependent, TUDCA/KIRA6-sensitive pathway, which differs from the TUDCA/KIRA6-resistant pathways induced by bacterial peptidoglycan fragments (Extended Data Fig. 1a). This finding provides a new link between NOD1, NOD2 and inflammatory diseases involving ER stress, such as Crohn disease, ulcerative colitis, obesity and type 2 diabetes.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Author Contributions** A.M.K.-G. and M.X.B. performed and analysed the experiments. R.R., P.A.L., O.H.P., A.Y.T., S.A.C., C.R.T., N.B.S., B.M.Y., A.C.-A., T.K., M.F.d.J. and M.G.W. performed experiments. A.M.K.-G., M.X.B., S.J.M., A.J.B. and R.M.T. were responsible for the overall study design and for writing the manuscript.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were blinded to allocation of mice for assessment of histopathology and readouts of inflammation.

Bacterial strains, tissue culture cells and culture conditions. E. coli strains were routinely cultured aerobically at 37°C in lysogeny broth (LB) and on LB agar plates. B. abortus was cultured in tryptic soy broth or on tryptic soy agar (TSA) plates. Chlamydia muridarum strain Nigg II was purchased from ATCC (Manassas, VA). Bacteria and C. muridarum were cultured in DMEM supplemented with 10% FBS. Elementary bodies (EBs) were purified by discontinuous density gradient centrifugations as described previously27 and stored at −80°C. The HEK293 cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS at 37°C in a 5% CO2 atmosphere.

Transfections. HEK293 cells (ATCC CRL-1573) were obtained from ATCC and were grown in a 48-well tissue culture plates in DMEM containing 10% FBS until ~40% of confluence was reached. HEK293 cells were transfected with a total of 250 ng of plasmid DNA per well, consisting of 25 ng of the reporter construct pNF-κB-Luc, 25 ng of the normalization vector pTK-LacZ, and 200 ng of the different combinations of mammalian expression vectors carrying the indicated gene (empty control vector, pCMV-HA-VeceC, pCMV-HA-TRA2FDN (this study), hNOD1-3×Flag, hNOD2-3×Flag, pCMV-HA-hRip2p, hNOD1DN-3×Flag, hNOD2DN-3×Flag or pCMV-HA-Rip2pDN3 and pCMV-myc-CDC42DN3). The dominant-negative form of TRAF2, lacking an amino-terminal RING finger domain28, was PCR amplified from cDNA prepared from HEK293 cells and cloned into the mammalian expression vector pCMV-HA (BD Biosciences Clontech). Forty-eight hours after transfection, cells were lysed either without any treatment, or stimulated with C12-iE-AP (1,000 ng ml⁻¹, InvivoGen) and MDP (10 μg ml⁻¹, InvivoGen). After five hours of treatment the cells were lysed and analysed for γ-galactosidase and a luciferase activity (Promega). Concentration of 2.5 μg plasmid DNA was used as a transfection reagent according to the manufacturer’s instructions. Cell lines were monitored for mycoplasma contamination.

Bone-marrow-derived macrophage infection. Bone-marrow-derived macrophages (BMDMs) were differentiated from bone marrow precursors from femur and tibiae of C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME), Nod1+/− Nod2+/− (wild-type littermates) and Nod1−/− Nod2−/− (NOD1/NOD2-deficient) mice (generated at UC Davis) as described previously27. For BMDM experiments, 24-well microtitre plates were seeded with macrophages at a concentration of 5 × 10⁶ cells per well in 0.5 ml of RPMI media (Invitrogen, Grand Island, NY) supplemented with 10% FBS and 100 μM l-glutamine (complete RPMI) and incubated for 48 h at 37°C in 5% CO2. BMDMs were stimulated with C12-iE-AP (1,000 ng ml⁻¹, InvivoGen), MDP (10 μg ml⁻¹, InvivoGen), thapsigargin (1 μg ml⁻¹, InvivoGen) and 10 μg ml⁻¹ l-glutamine and analysed for γ-galactosidase and luciferase activity (Promega). Concentration of 2.5 μg plasmid DNA was used as a transfection reagent according to the manufacturer’s instructions. Cell lines were monitored for mycoplasma contamination.

Real-time PCR. RNA was isolated from BMDMs and mouse tissues using Tri-reagent (Molecular Research Center) according to the instructions of the manufacturer. Reversetranscription was performed on 1 μg of DNase-treated RNA with Taqman reverse transcription reagent (Applied Biosystems). For each real-time reaction, 4 μl of cDNA was used combined with primer pairs for mouse Actb, Hspa5 and Chop. Real time transcription-PCR was performed using Sybr green and an ABI 7900 RT–PCR machine (Applied Biosystems). The fold change in mRNA levels was determined using the comparative threshold cycle (Ct) method. Target gene transcription was normalized to the levels of 18S rRNA.

Cytokine detection. Cytokine levels in mouse serum and supernatants of infected BMDMs were measured using either a multiplex cytokine/chemokine assay (Bio-Plex 23-plex mouse cytokine assay; Bio-Rad), or via an enzyme-linked immunosorbent assay (IL-6 ELISA; eBioscience), according to the manufacturer’s instructions. Cytokine detection.

LDH release assay. Cytotoxicity was determined by using a LDH release assay in supernatant of BMDMs treated as described above. LDH release assay was performed using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega), following manufacturer’s protocol. The percentage of LDH release was calculated as follows:

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\text{Percentage of LDH release} = 100 \times \frac{\text{(absorbance reading of treated well – absorbance reading of untreated control)}}{\text{(absorbance reading of maximum LDH release control – absorbance reading untreated control)}}
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The kit-provided absorbance standards were used to construct a standard curve. 50 μl of lysate from each condition was used for LDH release assay. For the placentitis mouse model, C57BL/6, Nod1+/− Nod2+/− and Nod1−/− Nod2−/− mice, aged 8–10 weeks, were kept in micro-isolator cages with sterile bedding and irradiated feed in a biosafety level 3 laboratory. Female Nod1+/− Nod2−/− mice were mated with male C57BL/6 mice (control mice) and female Nod1−/− Nod2−/− mice were mated with male Nod1−/− Nod2−/− mice (NOD1/NOD2-deficient), and pregnancy was confirmed by presence of a vaginal plug. At 5 days of gestation, groups of pregnant mice were mock infected or infected i.p. with 1 × 10⁵ CFU of Brucella abortus 2308 or its isogenic mutant ΔvceC (day 0). At 3, 7 and 13 days after infection mice were euthanized by CO2 asphyxiation and the spleen and placenta of dams were collected aseptically at necropsy. At day 13 after infection (corresponding to day 18 of gestation), viability of pups was evaluated based on the presence of fetal movement and heartbeat, and fetal size and skin colour. Fetuses were scored as viable if they exhibited movement and a heartbeat, visible blood vessels, bright pink skin, and were of normal size for their gestational period. Fetuses were scored as non-viable if fetal movement, heartbeat, and visible blood vessels were absent, skin was pale or opaque, and their size for gestational period or compared to littermates was small, or they showed evidence of fetal reabsorption. Percentage of viability was calculated as [(number viable pups per litter/total number pups per litter) × 100]. At each time point, the placenta samples were collected for bacteriology, gene expression analysis and blinded histopathological analysis (Extended Data Fig. 6d). When indicated, mice were treated i.p. at days 5, 7 and 9 post-infection with a daily dose of 250 mg per kg body weight of the ER stress inhibitor TUDCA (Sigma–Aldrich), or 10 mg per kg body weight of the IRE1α kinase inhibitor KIRA6 (Calbiochem) or vehicle control.

For the placentitis mouse model, C57BL/6, Nod1+/− Nod2+/− and Nod1−/− Nod2−/− mice were grown in a 48-well tissue culture plates in DMEM containing 10% FBS until 3–5 days of gestation. Nod2+/− mice were injected i.p. with 0.2 ml of PBS containing 5 × 10⁵ CFU of B. abortus 2308 or its isogenic mutant ΔvceC, as previously described28. At 3 days post-infection, mice were euthanized by CO2 asphyxiation and their serum and spleens were collected aseptically at necropsy. The spleens were homogenized in 2 ml of PBS, and serial dilutions of the homogenate were plated on TSA for enumeration of CFU. Spleen samples were also collected for gene expression analysis as described below. When necessary, mice were treated i.p. at day one and two post-infection with a daily dose of 250 mg per kg body weight of the ER stress inhibitor TUDCA (Sigma–Aldrich), or 10 mg per kg body weight of the IRE1α kinase inhibitor KIRA6 (Calbiochem) or vehicle control.

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supplemented with 10% FBS. HeLa 229 cells were transfected with a total of 125 ng of pCMV-HA-Rip2DN or empty control vector per well. 24 h post-transfection HeLa 229 cells were treated with Dextran to enhance infection efficacy before they were infected with $1.7 \times 10^5$ Chlamydia bacteria per well. The plates were centrifuged at 2,000 r.p.m. for 60 min at 37 °C, then incubated for 30 min at 37 °C in 5% CO₂. Supernatant was discarded and replaced with DMEM containing 1 μg ml⁻¹ cyclohexine (Sigma Aldrich) and where indicated, 1 μM KIRA6, 10 μM thapsigargin or 10 μg ml⁻¹ MDP, was added to cultures before incubation at 37 °C in 5% CO₂ for 40 h. For gene expression assays, HeLa 229 cells were suspended in Tri-reagent (Molecular Research Center, Cincinnati) and RNA was isolated. Infection efficiency was confirmed in separate plates by staining Chlamydia-infected HeLa 229 cells with anti-Chlamydia MOMP antibody and counting bacteria under a fluorescent microscope. Four independent assays were performed and the standard error of the mean calculated.

Western blots. BMDMs stimulated where indicated with 10 μM thapsigargin for 24 h were lysed in lysis buffer (4% SDS, 100 mM Tris, 20% glycerol) and 10 μg of protein was analysed by western blot using antibodies raised against rabbit TRAF2 (C192, #4724, Cell Signaling), rabbit HSP90 (E289, #4875, Cell Signaling), mouse SGT1 (ab60728, Abcam) and rabbit α/β-tubulin (#2148, Cell Signaling).

Statistical analysis. For tissue culture experiments, statistical differences were calculated using a paired Student’s t-test. To determine statistical significance in animal experiments, an unpaired Student’s t-test was used. To determine statistical significance of differences in total histopathology scores, a Mann–Whitney U-test was used. A two-tailed P value of < 0.05 was considered to be significant.

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Extended Data Figure 1 | Schematic of ER stress and NOD1/2 signalling. a, Model of how ER stress induces a NOD1/2-dependent pro-inflammatory response through a TUDCA/KIRA6-sensitive pathway, which differs from the TUDCA/KIRA6-resistant pathways induced by bacterial peptidoglycan fragments (MDP or C12-iE-DAP). b, NF-κB activation induced by ectopic expression of VceC in HEK293 cells transfected with a dominant negative form of TRAF2 or a vector control. c, NF-κB activation mediated by expression-induced auto-activation of NOD1, NOD2 or RIP2 in HEK293 cells that were transfected with a dominant negative form of TRAF2 or a vector control. Data are expressed as mean luciferase activity ± s.e.m. from five independent experiments.
Extended Data Figure 2 | See next page for caption.
Extended Data Figure 2 | Only the pro-inflammatory arm of the UPR requires NOD1 and NOD2. a, b, BMDMs from Nod1/2−/− mice and wild-type littermates were stimulated with thapsigargin or MDP, and mRNA abundance for Hspa5 (a) and Chop (b) was quantified (n = 4). c, Expression of SGT1, HSP90 and TRAF2 was detected by western blot in lysates of thapsigargin-stimulated BMDMs from wild-type mice and Nod1/2−/− mice (n = 3 mice of each genotype). Detection of tubulin served as a loading control. A representative image for BMDMs from one wild-type and one Nod1/2−/− animal is shown. d, LDH release induced by treatment of BMDMs from wild-type mice and Nod1/2−/− mice (n = 5) with thapsigargin, DTT or KIRA6. e, Stimulation with MDP or DTT induced IL-6 production in BMDMs from C57BL/6 mice (wild type) but not in BMDMs from Nod1/2−/− mice (n = 8). f, IL-6 secretion induced by thapsigargin, but not by the canonical NOD2 ligand MDP, was significantly inhibited by ER stress inhibitor TUDCA in BMDMs (n = 8). BMDMs did not respond to stimulation with a canonical NOD1 ligand (C12-iE-DAP). g–k, BMDMs from wild-type mice and Nod1/2−/− mice (n = 4) were treated with the PERK inhibitor GSK2656157 (GSK) (g, i) or the IRE1α RNase inhibitor STF-083010 (STF) (h, k) and IL-6 synthesis measured by ELISA (g, h) or mRNA analysed by real-time PCR (i, j). Data are presented as mean ± s.e.m. n represents the number of independent assays (biologic replicates) performed for each experiment.
Extended Data Figure 3 | Proinflammatory responses induced by thapsigargin are NOD1/2-dependent. a–c, Groups (n = 5) of wild-type mice and Nod1/2−/− mice were treated with thapsigargin and received either vehicle control or TUDCA. Synthesis of IL-6 (a), KC (b) and MIP-1β (c) in the serum was determined using a Bio-plex cytokine assay. d, e, Wild-type (C57BL/6) mice and Nod1/2−/− mice (n = 4) were treated with thapsigargin and transcript levels of Il6 determined by quantitative real-time PCR. Data are expressed as fold-increases over vehicle control-treated animals. Data are presented as mean ± s.e.m. n represents the number of independent assays (biologic replicates) performed for each experiment.
Extended Data Figure 4 | *B. abortus*-induced inflammatory responses in mice are blunted by TUDCA treatment. a–g. Mice (n ≥ 4) were mock infected or infected with the *B. abortus* wild type and were treated with TUDCA or vehicle control. Three days after infection, circulating levels of IL-6 (a), IL-12p40 (b), IFNγ (c), KC (d), MIP-1β (e), G-CSF (f) and RANTES (g) were profiled in serum using a Bio-Plex cytokine assay. Data are presented as mean ± s.e.m.
Extended Data Figure 5 | Bacterial burden and host responses during infection with *B. abortus*. a, c, e, g, i, Bacterial burden in the spleen and in BMDMs of wild-type and Nod1/2−/− mice (a, c, e, g) or Rip2−/− mice (i). No statistically significant differences in colony-forming units (CFU) recovered from the spleen (a, c, g, i) or from BMDMs (e) of wild-type and Nod1/2−/− or Rip2−/− mice (n ≥ 4) infected with *B. abortus* wild type or the vceC mutant were observed. b, d, f, h, Host responses elicited during *B. abortus* infection. b, Groups of mice (n = 5) were infected with the indicated *B. abortus* strains and treated with KIRA6. d, f, BMDMs from wild-type mice and Nod1/2−/− mice (d) or wild-type mice and Rip2−/− mice (n ≥ 4) (f) were infected with the indicated *B. abortus* strains. b, Groups (n = 5) of wild-type mice and Rip2−/− mice were infected with the indicated *B. abortus* strains. Il6 mRNA levels were determined by quantitative real-time PCR (b, d, h). IL-6 synthesis was determined by ELISA (f). Data are presented as mean ± s.e.m.
Extended Data Figure 6 | The B. abortus placentitis model. a, Bacterial numbers of wild-type B. abortus (strain 2308) recovered from in the spleen and placenta (n = 5 mice per group). b, c, Il6 mRNA expression (b) and total histopathology scores (c) in the placenta of mice at days 3, 7 and 13 after infection with B. abortus. d, Scoring criteria for blinded evaluation of haematoxylin and eosin (H&E)-stained sections from the placenta. e, Representative images of the histopathology observed in the placenta of B. abortus infected mice at days 3, 7 and 13 after infection. Arrow, neutrophil infiltration; N, necrosis.
Extended Data Figure 7 | Bacterial burden in the spleen and placenta of wild-type and Nod1/2−/− mice. a–c, No statistically significant differences in colony-forming units in the spleen and placenta of wild-type and Nod1/2−/− mice infected with B. abortus wild type or the vceC mutant at 13 days post-infection were observed. Data are presented as mean ± s.e.m. (n = 5 mice per group).
Extended Data Figure 8 | Il6 expression induced by Chlamydia muridarum. HeLa cells were stimulated with MDP, thapsigargin or infected with Chlamydia muridarum and treated with KIRA6 or transfected with RIP2DN (dominant negative form of RIP2). Expression of Il6 was determined by quantitative real-time PCR. Data are presented as mean ± s.e.m. from 4 independently performed assays.