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Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3

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Toll-like receptors (TLRs) are a family of innate immune-recognition receptors that recognize molecular patterns associated with microbial pathogens, and induce antimicrobial immune responses[1,2]. Double-stranded RNA (dsRNA) is a molecular pattern associated with viral infection, because it is produced by most viruses at some point during their replication[3]. Here we show that mammalian TLR3 recognizes dsRNA, and that activation of the receptor induces the activation of NF-κB and the production of type I interferons (IFNs). TLR3-deficient ($TLR3^{-/-}$) mice showed reduced responses to polyinosine–polycytidylic acid (poly(I:C)), resistance to the lethal effect of poly(I:C) when sensitized with D-galactosamine (D-GalN), and reduced production of inflammatory cytokines. MyD88 is an adaptor protein that is shared by all the known TLRs[1]. When activated by poly(I:C), TLR3 induces cytokine production through a signalling pathway dependent on MyD88. Moreover, poly(I:C) can induce activation of NF-κB and mitogen-activated protein (MAP) kinases independently of MyD88, and cause dendritic cells to mature.

Viral infection of mammalian cells results in activation of an innate immune response mediated by type I IFNs, IFN-α and IFN-β, and other cytokines, including interleukin (IL)-6 and IL-12 (refs 4, 5). While IFNs inhibit virus replication, IL-6 and IL-12, which are also induced by bacterial infections, elicit cytotoxic responses needed for elimination of intracellular pathogens. Mammalian TLRs recognize lipopolysaccharide (LPS) and other microbial products[1,6–10]. Whereas the receptors for LPS are expressed on the cell surface, dsRNA is known to bind only intracellular targets, including the dsRNA-dependent protein kinase (PKR)[11]. However, cells derived from PKR-deficient ($PKR^{-/-}$) mice still respond to poly(I:C), a synthetic dsRNA analogue, suggesting the existence of another receptor, which recognizes dsRNA[12,13].

To test whether dsRNA can be recognized by a TLR, 293T cells

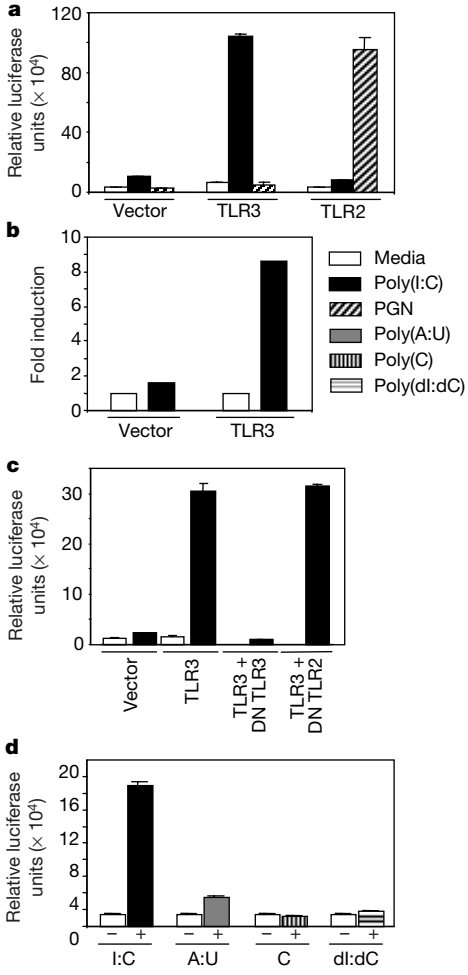

Figure 1 TLR3 specifically signals for NF-κB activation in response to poly(I:C). **a**, 293T cells were transiently transfected with 50 ng of human TLR3, TLR2 or empty pcDNA3 vector together with an NF-κB luciferase reporter. Luciferase activity in cells treated with 25 μg ml^{-1} poly(I:C) or 10 μg ml^{-1} PGN or untreated (media) cells was measured. **b**, Luciferase activity in CaCo-2 cells transiently transfected with 500 ng of empty vector or TLR3 DNA, together with 200 ng NF-κB luciferase reporter and stimulated with 25 μg ml^{-1} poly(I:C). **c**, 293T cells transiently transfected with expression vector for TLR3 or empty vector, together with NF-κB luciferase reporter and, where indicated, 1 μg of dominant negative (DN) TLR3 or DN TLR2 DNAs. NF-κB-induced luciferase activity in cells treated with 25 μg ml^{-1} poly(I:C) or untreated cells was measured. **d**, Transfection of RAW 264.7 macrophages with a NF-κB luciferase reporter. Luciferase activity in cells treated with 20 μg ml^{-1} poly(I:C), poly(A:U), poly(C) or poly(dI:dC), or untreated cells.

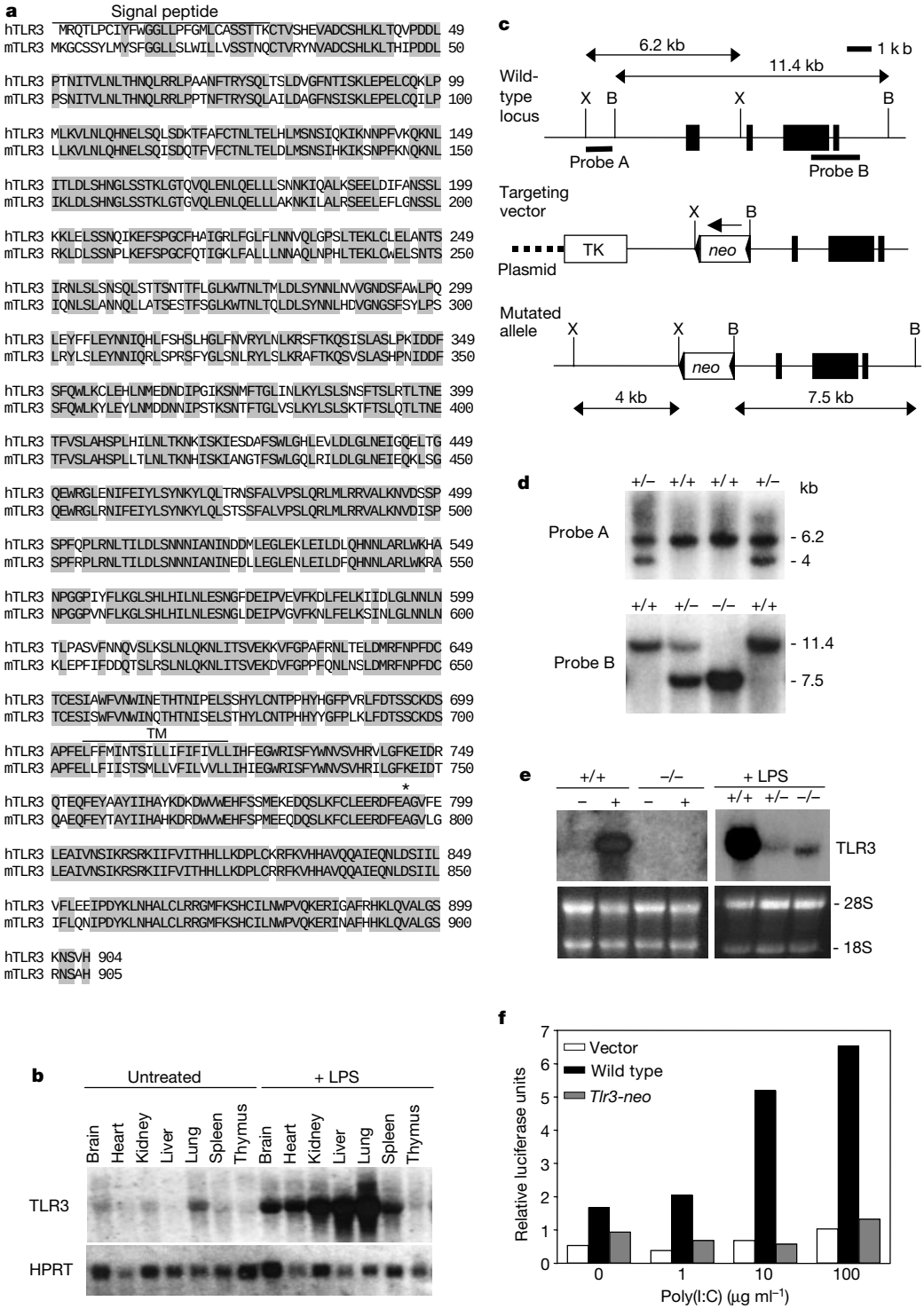

Figure 2 Amino-acid sequence and expression pattern of TLR3, and generation of TLR3$^{-/-}$ mice. **a**, Comparison of the human (h) and murine (m) (GenBank accession number AF355152 and AF40279, respectively) TLR3 sequences. Areas of amino-acid identity (79%) are shaded. The predicted signal peptide and transmembrane domain (TM) are indicated. Asterisk, alanine residue represented by a conserved proline residue in the other TLRs. **b**, Northern blot analysis of murine TLR3. Total RNA extracted from multiple tissues of mice killed 0 or 4 h after intraperitoneal LPS (500 μg) injection was electrophoresed, transferred to a nylon membrane, and hybridized with a 980-bp *Sma*I–*Xba*I fragment, containing part of the third exon of *Tlr3* gene as a probe. **c**, The structure of the *Tlr3* gene, the targeting vector and the predicted mutated locus. Filled boxes are exons, filled triangles are *loxP* sites, and open boxes are the selection marker genes. The locations of the 5′ external and 3′ internal probes are shown. Restriction enzymes: B, *Bam*HI; X, *Xba*I. TK, thymidine kinase gene. **d**, Southern blot analysis of genomic DNA from embryonic stem-cell clones and from mice digested with *Xba*I and *Bam*HI, respectively. DNA from wild-type (+/+) and targeted embryonic stem (+/–) clones (top), and from wild-type (+/+), heterozygous (+/–) and homozygous (–/–) TLR3 mutant mice (bottom) are indicated. **e**, Northern blot analysis of RNA from thioglycollate-elicited uninduced (–) or LPS-induced (+) macrophages from wild-type (+/+), heterozygous (+/–) and homozygous (–/–) TLR3 mutant mice. Hybridization using the TLR3 N-terminal (left) or C-terminal (right) fragment as a probe is shown. Ethidium bromide staining after RNA transfer to the membrane is included as control (bottom panels). **f**, Wild-type *Tlr3* but not *Tlr3-neo* activates NF-κB. 293T cells were transiently transfected with wild-type *Tlr3*, *Tlr3-neo* or empty expression vectors together with a NF-κB luciferase reporter. Luciferase activity is expressed as the ratio of NF-κB-dependent firefly luciferase activity divided by control *Renilla* luciferase activity. Data are representative of two independent experiments.

that do not respond to stimulation with poly(I:C) (Fig. 1a) were used in an *in vitro* system. We used 293T cells expressing one of a range of TLRs (human TLR1–6 or TLR9) together with an NF-κB-dependent reporter gene. When stimulated with poly(I:C), only those cells expressing human TLR3 showed marked responsiveness to poly(I:C); the cells expressing the rest of the TLRs, including TLR2, did not (Fig. 1a and data not shown). To demonstrate the specificity of the ligand, we used peptidoglycan (PGN), which activated TLR2, as previously reported[9], but not TLR3 (Fig. 1a). Similar results were obtained when we used the human Caco-2 cells, which also do not respond to poly(I:C) (Fig. 1b). Moreover, co-transfection of a dominant negative version of TLR3 in 293T cells,

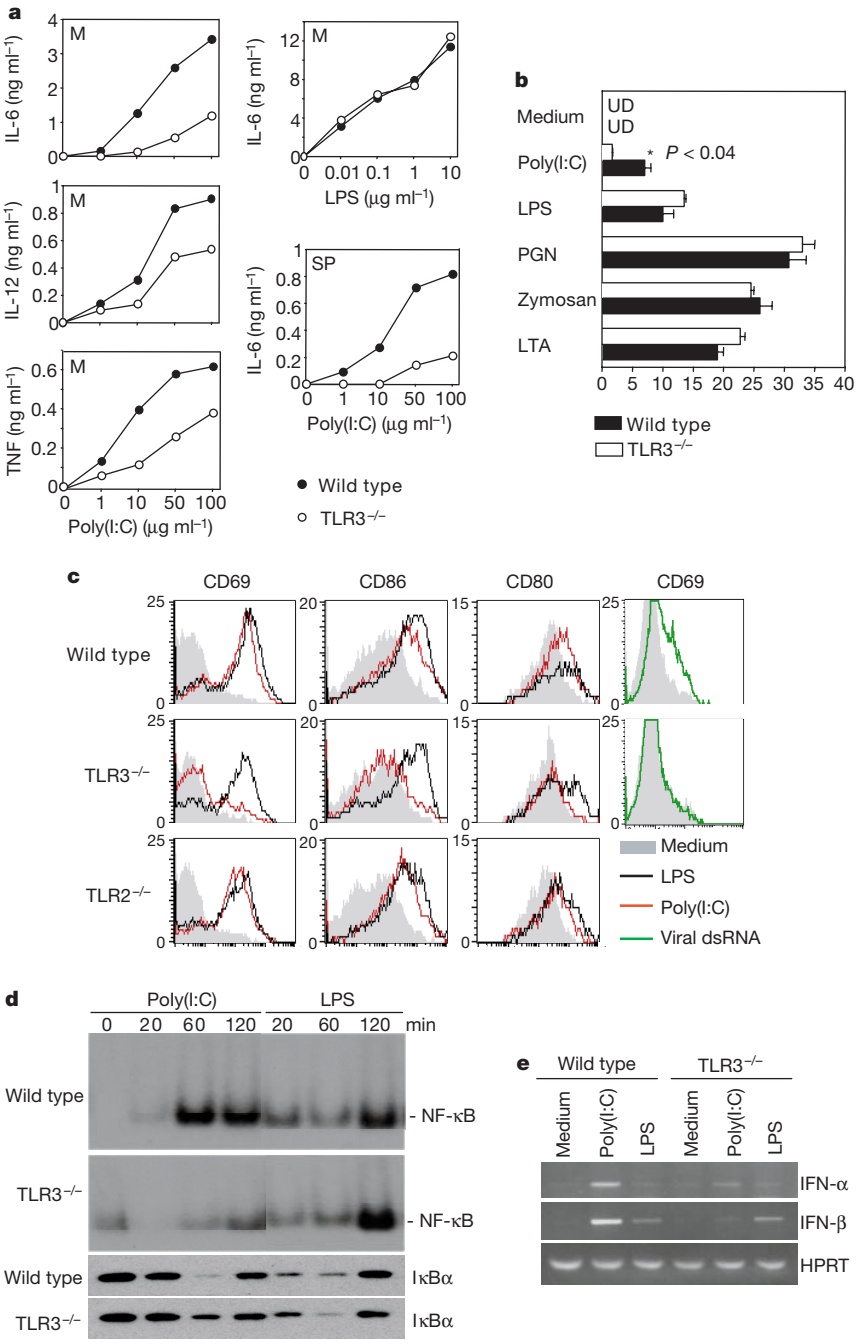

Figure 3 Impaired responses to poly(I:C) from TLR3$^{-/-}$ cells. **a**, Bone-marrow-derived macrophages (M) or total splenocytes (SP) from wild-type or TLR3$^{-/-}$ mice were stimulated with poly(I:C) or LPS for 24 h, and concentrations of IL-6, IL-2 p40/p70 and TNF-α in the culture supernatants were measured by ELISA. Data are representative of three independent experiments. **b**, Bone-marrow-derived macrophages from wild-type or TLR3$^{-/-}$ mice were stimulated with 100 μg ml^{-1} poly(I:C), 10 μg ml^{-1} LPS, 10 μg ml^{-1} PGN, 100 μg ml^{-1} zymosan or 10 μg ml^{-1} LTA for 24 h, and concentrations of IL-6 in the culture supernatants were measured by ELISA. Data are representative of two independent experiments. UD, undetected. **c**, Splenocytes were cultured for 24 h with 100 μg ml^{-1} poly(I:C), 5 ng ml^{-1} LPS or 30 μg ml^{-1} viral dsRNA, or left untreated. Cells were collected, stained and analysed by flow cytometry. Histograms show expression levels of CD69, CD86 and CD80 in B220^{+} (B cells) gated lymphocyte populations. **d**, Bone-marrow-derived macrophages from wild-type or TLR3$^{-/-}$ mice were stimulated with 20 μg ml^{-1} poly(I:C) or 5 ng ml^{-1} LPS. At the indicated points, cells were lysed; nuclear translocation of NF-κB was visualized by EMSA, and degradation of Iκ-Bα was analysed by western blot. **e**, Bone-marrow-derived macrophages from wild-type or TLR3$^{-/-}$ mice were stimulated with 100 μg ml^{-1} poly(I:C) or 1 μg ml^{-1} LPS, or left untreated. After 4 h, total RNA was isolated and expression of IFN-α, IFN-β and HPRT was determined by RT-PCR.

but not of a dominant negative TLR2, completely blocked poly(I:C) signalling (Fig. 1c), indicating that TLR3 confers responsiveness to poly(I:C).

Treatment of a RAW 264.7 macrophage cell line with poly(I:C) resulted in activation of the NF-κB reporter gene (Fig. 1d). Additionally, polyadenylic–polyuridylic acid (poly(A:U)), which is another dsRNA polymer, activated RAW macrophages in a manner similar to poly(I:C), but significantly less strongly (Fig. 1d). Importantly, neither polycytidylic acid (poly(C)), a synthetic single-stranded RNA analogue, nor polydeoxyinosinic–deoxcytidylic acid (poly(dI:dC)) had any effect on NF-κB activation in RAW cells (Fig. 1d), as expected.

To characterize the biological function of murine TLR3, we generated TLR3$^{-/-}$ mice by homologous recombination in embryonic stem cells. To isolate the murine TLR3 gene, we screened a 129/SvJ mouse genomic library with a 980-base pair (bp) complementary DNA fragment of human TLR3 as a probe. Sequence analysis revealed the presence of regions conserved in the TLR family, such as leucine-rich repeats (LRRs) and a Toll/IL-1 receptor (TIR) homology domain (Fig. 2a). However, the conserved proline residue within the cytoplasmic domain of the murine and human TLR proteins that is mutated in LPS-hyporesponsive C3H/HeJ mice (P712H)[14] is represented by an alanine residue in TLR3 (Fig. 2a). Murine TLR3 transcripts were most abundantly expressed in the lung, brain and kidney as detected by northern blot analysis (Fig. 2b). However, when LPS was injected intraperitoneally, a dramatic upregulation of expression of *TLR3* messenger RNA could be seen in all tissues tested except thymus, suggesting that the expression of TLR3 is inducible (Fig. 2b).

To generate TLR3$^{-/-}$ mice, we replaced the first exon of the *Tlr3* gene with a neomycin-resistance cassette (*neo*) flanked by two *loxP* sites (Fig. 2c). Chimaeric mice were produced by microinjecting embryonic stem cells from three correctly targeted clones into C57BL/6 blastocysts. Male chimaeras were mated to C57BL/6 females and the transmission of the mutated allele through the germ line was confirmed in all three lines by Southern blot analysis (Fig. 2d). Northern blot analysis showed that *Tlr3* transcript from the mutant mice was absent or detected as smaller and in reduced amounts compared with that from the wild-type mice, when an amino-terminal or a carboxy-terminal fragment was used as a probe, respectively (Fig. 2e). Because low levels of a truncated *Tlr3* mRNA were detected, a transient transfection assay was used to assess the functional activities of wild-type (*Tlr3*) and mutant *Tlr3* (*Tlr3-neo*) genes. The genes were cloned into an expression vector and transiently transfected into 293T cells together with an NF-κB luciferase reporter gene. When the cells were stimulated with poly(I:C), substantial activation of NF-κB was observed only in the wild-type *Tlr3* cells (Fig. 2f), indicating that the truncated transcript *Tlr3-neo* expressed in the mutant mice does not encode a functional protein.

TLR3$^{-/-}$ mice had normal appearance, growth, size, fertility and lifespan, and showed no obvious behavioural abnormalities. Moreover, flow cytometry revealed that the expression of CD3, B220, CD4 and CD8 in thymocytes and splenocytes were not altered in TLR3$^{-/-}$ mice compared with wild-type mice (data not shown).

Our *in vitro* experiments demonstrated that stimulation of TLR3 by poly(I:C) leads (directly or indirectly) to the activation of NF-κB (Fig. 1). We therefore analysed responses of TLR3$^{-/-}$ cells to poly(I:C). Macrophages from wild-type mice produced IL-6, IL-12 and TNF-α in a dose-dependent manner in response to poly(I:C) (Fig. 3a). However, the ability of TLR3$^{-/-}$ macrophages to produce these inflammatory cytokines in response to poly(I:C) was significantly impaired (Fig. 3a). Moreover, the production of IL-6 from TLR3$^{-/-}$ total splenocytes was impaired compared with that of wild-type cells (Fig. 3a). No significant differences were observed in the production of TNF-α, IL-6 or IL-12 between wild-type and TLR3$^{-/-}$ macrophages in response to LPS, PGN, lipoteichoic acid (LTA), zymosan, mannan or CpG DNA (Fig. 3b and data not shown), indicating that there were no detectable intrinsic defects in cytokine production in TLR3$^{-/-}$ macrophages other than their specific defect in their response to poly(I:C).

To demonstrate the specificity of recognition of poly(I:C) by TLR3, we tested the activation of B cells by TLR3$^{-/-}$ cells and compared it with that by TLR2-deficient (TLR2$^{-/-}$)[9] and wild-type cells. Total splenocytes from wild-type, TLR2$^{-/-}$ and TLR3$^{-/-}$ mice were stimulated with poly(I:C) or LPS and the expression of CD69, CD80 and CD86 on B cells was analysed by flow cytometry (Fig. 3c). A comparable augmentation of the expression of these surface molecules was observed in wild-type and TLR2$^{-/-}$ B cells (Fig. 3c). In contrast, TLR3$^{-/-}$ B cells did not show any response to poly(I:C), despite exhibiting normal responses to LPS (Fig. 3c), indicating that TLR3 is important for cellular responses to poly(I:C) but not to LPS. Similar results were obtained with macrophages (data not shown). We then tested the responses of TLR3$^{-/-}$ and wild-type splenocytes when stimulated with viral genomic dsRNA from type I Lang mammalian reovirus. In contrast to wild-type B cells, which upregulated CD69, TLR3$^{-/-}$ cells showed no response to viral dsRNA (Fig. 3c), suggesting that TLR3 is important for recognizing not only synthetic poly(I:C), but also viral dsRNA.

Signalling pathways that are responsive to viral infection or dsRNA involve activation of NF-κB and c-Jun N-terminal kinase (JNK)[5,12,13]. We next analysed poly(I:C)-induced NF-κB activation at different time points by electrophoretic mobility shift assay. In wild-type macrophages, binding activity of NF-κB was detected at 60 min and sustained for up to 120 min. In contrast, in TLR3$^{-/-}$ macrophages, activation of NF-κB was barely detectable at 60 min,

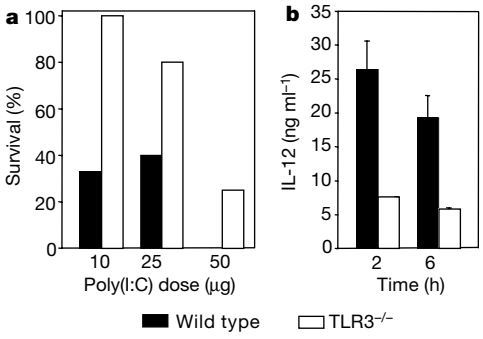

Figure 4 Measurement of poly(I:C)-induced shock in TLR3$^{-/-}$ mice. **a**, Mice (8–12 weeks old) were injected intraperitoneally with the indicated amounts of poly(I:C) and 20 mg D-GalN (Sigma). The dose of poly(I:C) was calculated per 20 g of body mass. Survival was monitored for 3 d. Three, five and four mice were used for 10, 25 and 50 μg poly(I:C), respectively. **b**, TLR3$^{-/-}$ and wild-type mice were injected intraperitoneally with 50 μg poly(I:C). Sera were taken 2 and 6 h later and serum levels of IL-12 were determined with specific ELISA assays. The data are means ± s.e. of sera samples from two and four mice at 2 and 6 h, respectively.

and was not increased even after 120 min (Fig. 3d). However, TLR3$^{-/-}$ macrophages stimulated by LPS activated NF-κB to the same extent as did wild-type cells (Fig. 3d). Analysis of the levels of IκBα protein in cytoplasmic extracts yielded similar results (Fig. 3d).

Type I IFNs are induced by virtually every type of viral infection, at least in part through the detection of viral dsRNA, and mediate their antiviral activities through the induction of cellular proteins that in turn participate in RNA degradation, inhibition of translation, and regulation of major histocompatibility complex (MHC) antigens[5]. To determine whether TLR3 contributes to induction of type I IFN genes, we used macrophages derived from wild-type and TLR3$^{-/-}$ mice. Little induction of IFN-α or IFN-β mRNA was observed in TLR3$^{-/-}$ macrophages when treated with poly(I:C) compared with wild-type cells (Fig. 3e). When stimulated with LPS, the induction of type I IFNs was much less pronounced, but was similar in wild-type and TLR3$^{-/-}$ macrophages (Fig. 3e). This result indicates that the transcriptional induction by dsRNA of genes for IFN-α and -β is dependent on TLR3.

Next, we investigated whether signalling by TLR3 is required for the *in vivo* inflammatory response to poly(I:C)[15]. TLR3$^{-/-}$ and wild-type mice were injected intraperitoneally with poly(I:C) upon D-GalN sensitization. TLR3$^{-/-}$ mice were significantly resistant to poly(I:C)-induced shock compared with wild-type mice (Fig. 4a). Moreover, when injected with poly(I:C), TLR3$^{-/-}$ mice exhibited reduced production of IL-12 in the sera compared with wild-type controls (Fig. 4b).

Members of the TLR family activate the Rel-family transcription factor NF-κB through the adapter protein MyD88, the serine/threonine kinase IRAK, and the tumour necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) (refs 1, 16). To test whether MyD88 is involved in dsRNA-induced signalling, we analysed poly(I:C)-induced maturation of dendritic cells and activation of macrophages derived from MyD88-deficient (MyD88$^{-/-}$) mice[17]. Stimulation of wild-type dendritic cells with poly(I:C) induced production of IL-12 (Fig. 5a) and IL-6 (data not shown) in a dose-dependent manner, whereas only background levels were detectable in MyD88$^{-/-}$ dendritic cells (Fig. 5a). Similar results were obtained with macrophages (data not shown). Moreover, MyD88$^{-/-}$ macrophages were also deficient in secreting NO_2 when treated with poly(I:C) or LPS (Fig. 5b). However, denatured poly(I:C) or single-stranded RNA, such as poly(C), did not activate wild-type or MyD88$^{-/-}$ macrophages (Fig. 5b) or dendritic cells (data not shown). Additionally, MyD88$^{-/-}$ total splenocytes showed only a

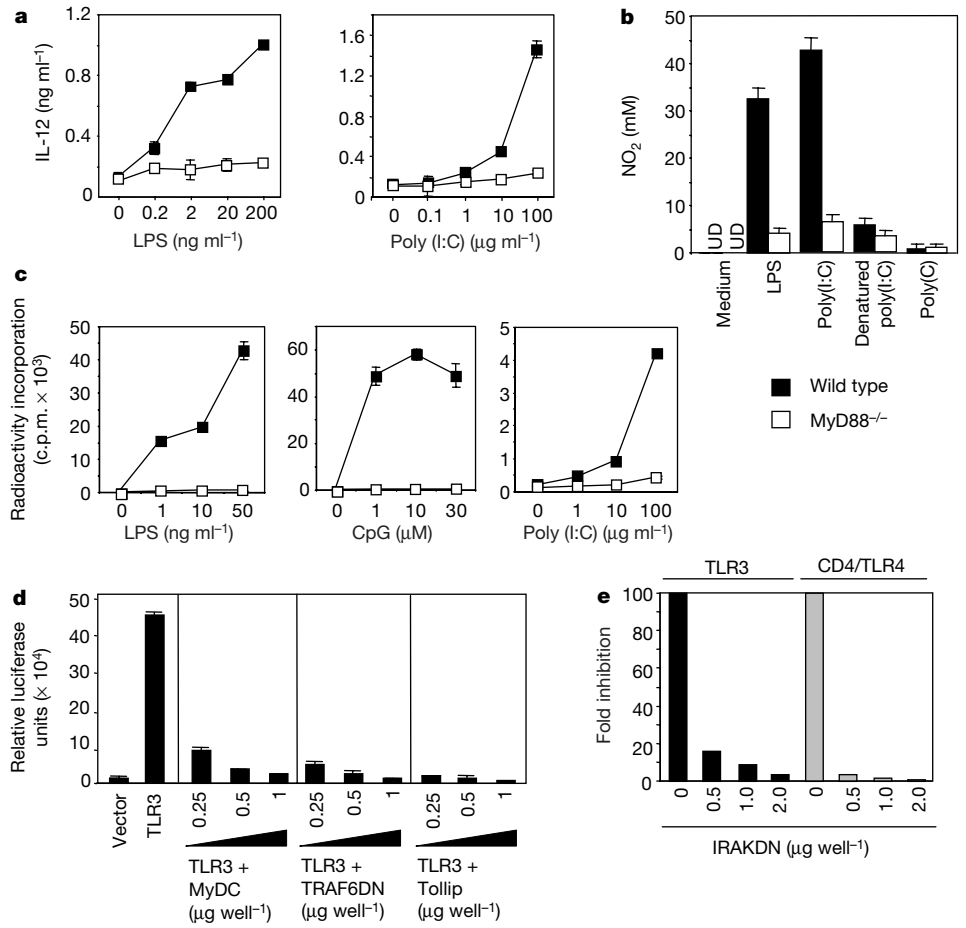

Figure 5 Responses to poly(I:C) in MyD88$^{-/-}$ and TLR3-transfected 293T cells. **a**, Bone-marrow-derived dendritic cells from wild-type or MyD88$^{-/-}$ mice were treated with increasing concentrations of LPS and poly(I:C). After 24 h, IL-12 in the culture supernatants was measured by ELISA. **b**, Bone-marrow-derived macrophages were stimulated with 20 ng ml^{-1} LPS, 20 μg ml^{-1} poly(I:C), 20 μg ml^{-1} denatured poly(I:C) or 20 μg ml^{-1} poly(C) for 24 h, and the concentration of NO_2 in the culture supernatants was measured. **c**, Total splenocyte suspensions (2×10^6 ml^{-1}) from wild-type or MyD88$^{-/-}$ mice were treated with the indicated amounts of LPS, CpG or poly(I:C) in RPMI, 10% FBS. After 48 h, cells were pulsed with [^{3}H]thymidine for 16 h and radioactivity incorporation was measured with a β-scintillation counter. **d**, 293T cells were transiently transfected as in Fig. 1a, including the indicated amounts of dominant negative (DN) constructs: MyDC, DN MyD88 with death-domain deletion; TRAF6DN, TRAF6 with deletion of N-terminal interaction domain; and full-length Tollip. Cells were left untreated or stimulated with 25 μg ml^{-1} poly(I:C) for 8 h (black bars) and luciferase activity was measured. **e**, 293T cells were transfected with TLR3 or a constitutively active CD4/TLR4 chimaera, together with an NF-κB luciferase reporter and the indicated amounts of DN IRAK (IRAKDN). Cells were stimulated with 25 μg ml^{-1} poly(I:C) for 8 h, and luciferase levels were measured.

minor increase in proliferation compared with wild-type cells when stimulated with poly(I:C) (Fig. 5c). As expected, neither LPS nor CpG induced proliferation of MyD88$^{-/-}$ splenocytes (Fig. 5c). Thus, MyD88 is involved in dsRNA-induced cellular responses.

We next examined the TLR3-induced signal transduction pathway activated by poly(I:C) treatment. Co-transfection of 293T cells with increasing amounts of dominant negative MyD88 or dominant negative TRAF6 together with a constant amount of TLR3 resulted in a dose-dependent inhibition of the stimulation induced by poly(I:C) (Fig. 5d). Moreover, overexpression of Tollip, a protein that interacts with the intracellular domain of the IL-1 receptor and IRAK[18], was also found to profoundly inhibit the poly(I:C)-induced activation of TLR3 (Fig. 5d). Finally, poly(I:C)-induced activation of TLR3 was inhibited by a double negative IRAK in a dose-dependent manner, similarly to the inhibition of signalling by CD4/TLR4, a constitutively active chimaera of TLR4 (ref. 19), which was used as a positive control (Fig. 5e). These results suggest that MyD88, IRAK, TRAF6 and Tollip are components of the TLR3-induced signalling pathway.

Previous findings reported by several groups have shown that poly(I:C) treatment of macrophages induces activation of NF-κB and MAP kinases[12,20]. We next analysed the degradation of IκBα and activation of MAP kinase in MyD88$^{-/-}$ cells in response to poly(I:C). The kinetics of IκBα degradation in MyD88$^{-/-}$ macrophages activated with poly I:C was similar to that in wild-type macrophages (Fig. 6a). By contrast, IκBα degradation was delayed in MyD88$^{-/-}$ cells treated with LPS or absent in cells treated with CpG (Fig. 6a), as previously reported[21,22]. Our analysis of stress-activated protein kinase (SAPK)/JNK and p38 MAP kinases demonstrated that poly(I:C) induced activation of JNK and p38 to a similar extent and with similar kinetics in wild-type and MyD88$^{-/-}$ cells (Fig. 6b). Phosphorylation of JNK and p38 MAP kinase in LPS-treated MyD88$^{-/-}$ macrophages was delayed, whereas in CpG-treated cells it was completely absent (Fig. 6b), consistent with previously published reports[17,21,22]. Thus, activation of NF-κB and MAP kinases in response to poly(I:C) can occur in the absence of MyD88.

We next tested the ability of MyD88$^{-/-}$ dendritic cells to mature in response to poly(I:C). Surprisingly, we found that poly(I:C)-induced maturation of dendritic cells, as measured by upregulation of MHC class II and CD86 molecules, was not impaired in MyD88$^{-/-}$ cells (Fig. 6c). Our results suggest that, in response to poly(I:C), TLR3 is involved in cytokine production via a MyD88-dependent pathway, whereas maturation of dendritic cells and activation of NF-κB and MAP kinases are induced via a MyD88-independent pathway. This dichotomy in signalling responses is similar to that observed for LPS signalling through TLR4 (ref. 23). However, it is not yet clear whether the MyD88-independent signalling induced by TLR4 and TLR3 relies on the same pathway.

We have shown here that TLR3 is critical in the recognition of dsRNA. The presence of viral dsRNA seems to trigger many of the cellular responses to viral infection, through activation of dsRNA-dependent enzymes including PKR, which inhibits viral protein synthesis, and the IFN-inducible 2′-5′-adenylate synthase/Rnase L system, which degrades viral RNA[3,24]. Both of these molecules are intracellular and can initiate the innate immune response when viral infection occurs. However, the fact that mice defective in PKR, and

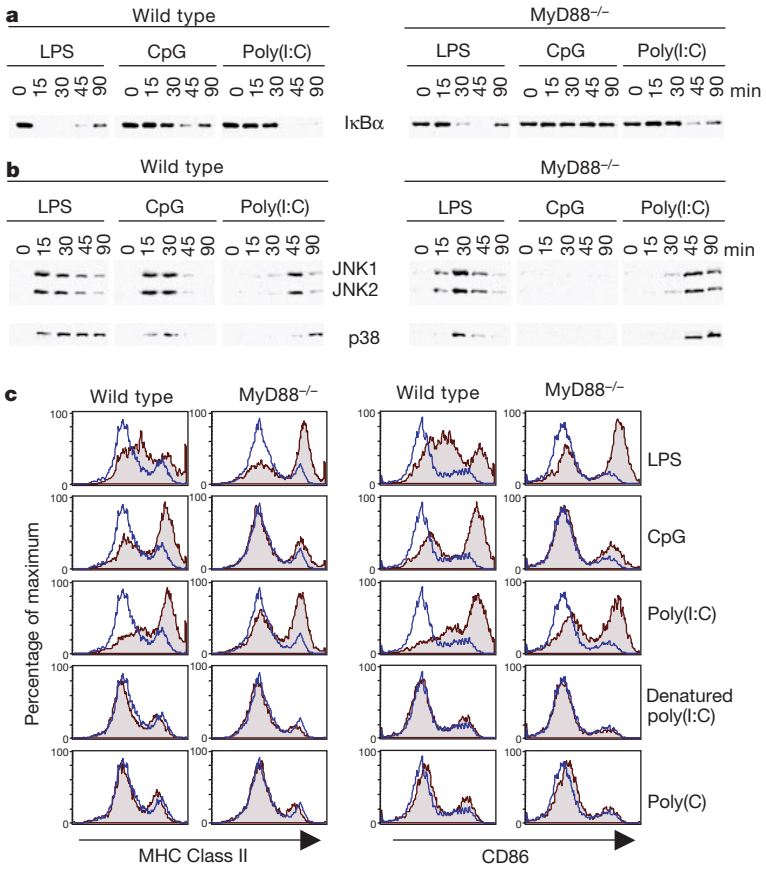

Figure 6 Cellular responses independent of MyD88. **a**, Macrophages from wild-type or MyD88$^{-/-}$ mice were stimulated with 20 ng ml^{-1} LPS, 10 μM CpG oligonucleotide or 20 μg ml^{-1} poly(I:C) for the indicated durations. Cell lysates were prepared and western blotting was performed with anti-IκBα antibody. **b**, The same lysates were blotted with phospho-specific antibodies for SAPK/JNK and p38 MAP kinases. **c**, Dendritic cells were stimulated with 10 ng ml^{-1} LPS, 10 μM CpG, 20 μg ml^{-1} poly(I:C), poly(C) or denatured poly(I:C) for 24 h and CD11c^{+} cells analysed for the cell surface expression of the indicated molecules by flow cytometry. White area, untreated dendritic cells; grey area, treated dendritic cells.

in both PKR and Rnase L, can still respond to dsRNA or viral infection, suggests that there are additional pathways of viral recognition[25–27]. Lysis of virally infected cells leads to the release of dsRNA, which then can be detected by the adjacent cells through a transmembrane receptor. Alternatively, after limited proteolysis, dsRNA-containing virions could be recognized in TLR3$^+$ vesicles. The importance of TLR3 in the antiviral response, however, remains to be established. Challenge of TLR3-deficient mice with a range of viruses will be required to elucidate whether TLR3 has a role in the host's defence against viruses. □

Methods

Generation of TLR3$^{-/-}$ mice

For the generation of TLR3$^{-/-}$ mice the genomic DNA of the *Tlr3* gene was isolated from a 129SV mouse genomic library (Stratagene). A targeting vector was constructed from a 3.0-kilobase (kb) *Nco*I–*Eco*RI fragment containing the 5′ end of the *Tlr3* gene and a 6.7-kb *Nco*I–*Xho*I fragment containing exons 2–4 and the 3′ end of the *Tlr3* gene. The first exon of the *Tlr3* gene, including the translation-initiation codon ATG and the signal peptide, was replaced by a *neo* cassette flanked by two *loxP* sites, and a thymidine kinase gene was used for negative selection of clones with random integration of the targeting vector (Fig. 2c). The targeting construct was transfected into embryonic stem cells (W9.5) using a standard protocol, and homologous recombinants were identified by Southern blot analysis (Fig. 2d). Thirteen *Tlr3*-targeted embryonic stem clones were identified and three of them used to generate chimaeric mice. Homozygous TLR3$^{-/-}$ and wild-type (TLR3$^{+/+}$) mice were generated by intercrossing F_1 heterozygous (TLR3$^{+/-}$) mice. The homozygous mice were then interbred for the current studies.

Reagents

Poly(I:C) and PGN from *Staphylococcus aureus* were purchased from Amersham and Fluka, respectively. Poly(A:U), poly(C), poly(dI:dC) and LTA from *S. aureus* and LPS from *Salmonella enteritidis* (used in Figs 2 and 3) or *Escherichia coli* (used in Figs 5 and 6) were purchased from Sigma. CpG oligonucleotides were from Keck, Yale University. Viral dsRNA from type I Lang mammalian reovirus was kindly provided by M. Nibert.

NF-κB assays

HEK 293T cells were transiently transfected using Lipofectamine 2000 reagent (Gibco BRL), according to the manufacturer's instruction, with the indicated amounts of expression plasmids and reporter pBIIXLuc plasmid. The total amount of transfected DNA was kept constant by adding empty vector (Fig. 1), or 293T cells were co-transfected with 5 ng Renilla luciferase (pRL-null, Promega) (Fig. 2f). Where indicated, cells were treated with poly(I:C), poly(A:U), poly(C), poly(dI:dC) or PGN. Cells were lysed 6–8 h after stimulation and luciferase activity was measured following the manufacturer's instructions. The Flag-tagged human TLR3 and TLR2 were cloned into pCMV1 Flag vectors. Dominant negative TLR3 and TLR2 have a deletion of the TIR domain. Dominant negative MyD88, IRAK, TRAF6 and CD4/TLR4 were as described previously[28]. The Tollip expression construct was created by inserting a cDNA fragment, amplified by polymerase chain reaction (PCR), into the pcDNA3 expression vector (Promega).

Analysis of macrophages and dendritic cells

Bone marrow cells were cultured in DMEM medium supplemented with 20% fetal bovine serum (FBS) and 30% supernatant derived from L929 confluent cells. At day 5 or 6, immature macrophages were collected and cultured in the presence or absence of stimuli in RPMI 1640 medium, 5% FBS. Bone marrow dendritic cells were prepared as described previously[29]. On day 5, adherent cells were stimulated or left untreated and on day 7 they were collected for analysis.

Measurement of cytokine production

Macrophages (1×10^6 cells ml^{-1}), dendritic cells (1×10^6 cells ml^{-1}) or total splenocytes (6×10^6 cells ml^{-1}) were cultured with the indicated stimuli for 24 h. Concentrations of IL-6, IL-12 p40/p70 (antibodies from PharMingen) and TNF-α (Duoset) in the cultured supernatants were measured by enzyme-linked immunosorbent assay (ELISA). NO_2 production was measured by the Griess assay.

Electrophoretic mobility shift assay (EMSA)

Macrophages from wild-type or TLR3$^{-/-}$ mice were stimulated for the indicated periods and then nuclear proteins were extracted. The extracts (3 g) were incubated for 30 min at room temperature with a specific ^{32}P-labelled probe containing NF-κB DNA-binding sites, electrophoresed on a 5% polyacrylamide gel and visualized by autoradiography.

PCR with reverse transcription (RT-PCR)

Total RNA from macrophages was isolated with TRIzol reagent (Gibco BRL), and contaminant DNA was removed by Dnasin (Ambion), according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed using Superscript reverse transcriptase (Gibco BRL) in a total volume of 20 μl. Five per cent of this reaction was used as a template for PCR amplification with Tsg DNA polymerase (LAMDA Biotech) for 25–30 cycles at 94 °C for 30 s, 54 °C for 40 s, and 72 °C for 40 min. The IFN-α, IFN-β and HPRT cDNAs were amplified with the following primers: 5′-CTCGTGATGCTGATAG TGATGAGC-3′ and 5′-CCACACTTTGTCTCACACTCACTCC -3′ for IFN-α, 5′-TTCCTGCTGTGCTTCTCCAC-3′ and 5′-GATTCACTACCAGTCCCAGAGTC-3′ for IFN-β, and 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and 5′-TCGGATTCCGGT CGGATGGGAG-3′ for HPRT.

Western blotting

Macrophages (5×10^5 cells ml^{-1}) were activated with the indicated stimuli and for the indicated time and lysed; total protein (80 μg) was resolved on 10% SDS–PAGE gels and transferred to Immobilon P membranes. Blotting was performed with anti-IκBα anibody (Santa Cruz Biotechnology), phospho-SAPK/JNK or phospho-p38 MAPK (New England Biolabs). Bands were visualized with secondary HRP-conjugated antibodies and the ECL System (Amersham Pharmacia).

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