Identification of *Lps2* as a key transducer of MyD88-independent TIR signalling

K. Hoebe[1], X. Du[1], P. Georgel[1], E. Janssen[2], K. Tabeta[1], S. O. Kim[1], J. Goode[1], P. Lin[1], N. Mann[1], S. Mudd[1], K. Crozat[1], S. Sovath[1], J. Han[1] & B. Beutler[1]

[1]*Department of Immunology, IMM-31, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA*
[2]*Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, California 92121, USA*

In humans, ten Toll-like receptor (TLR) paralogues sense molecular components of microbes, initiating the production of cytokine mediators that create the inflammatory response. Using *N*-ethyl-*N*-nitrosourea, we induced a germline mutation called *Lps2*, which abolishes cytokine responses to double-stranded RNA and severely impairs responses to the endotoxin lipopolysaccharide (LPS), indicating that TLR3 and TLR4 might share a specific, proximal transducer. Here we identify the *Lps2* mutation: a distal frameshift error in a Toll/interleukin-1 receptor/resistance (TIR) adaptor protein known as Trif or Ticam-1. *Trif*^{Lps2} homozygotes are markedly resistant to the toxic effects of LPS, and are hypersusceptible to mouse cytomegalovirus, failing to produce type I interferons when infected. Compound homozygosity for mutations at *Trif* and *MyD88* (a cytoplasmic TIR-domain-containing adaptor protein) loci ablates all responses to LPS, indicating that only two signalling pathways emanate from the LPS receptor. However, a Trif-independent cell population is detectable when *Trif*^{Lps2} mutant macrophages are stimulated with LPS. This reveals that an alternative MyD88-dependent 'adaptor X' pathway is present in some, but not all, macrophages, and implies afferent immune specialization.

During the past several years the TLRs have been shown to act as primary innate immune sensors, each responding to specific molecules of microbial origin[1–7]. They alert the host to the presence of infectious organisms within minutes after inoculation, and also initiate the systemic inflammatory state that accompanies sepsis[8], marked by the production of tumour-necrosis factor (TNF), interleukin (IL)-1, IL-6, IL-12, type I interferons and other proteins. All TLRs are single-spanning membrane proteins that display a conserved cytoplasmic motif (the TIR domain[9]), and it is thought that MyD88 acts as a transducer of signalling by all TLRs with the possible exception of TLR3. MyD88 signals by way of IRAK-4, a serine kinase required for the effective production of inflammatory cytokines induced by all of the TLRs[10,11]. But MyD88 is not the sole adaptor for TLR signalling. At least five TIR domain adaptors are encoded in the human genome, and some TLRs depend on more than one adaptor. The endotoxin (lipopolysaccharide; LPS) receptor, TLR4, provides an example.

As mice lacking MyD88 still exhibit some LPS responses, the existence of a 'MyD88-independent' pathway has been proposed[12]. One of the hallmarks of this pathway is the activation of interferon-β production[13], a signalling endpoint also known to be elicited by double-stranded (ds)RNA stimulation[14]. Probably as a result of interferon-β receptor stimulation and JAK/STAT activation, the production of nitric oxide (NO)[12] and the chemokine IP-10[13] are elicited in turn, and may also be considered as elements of the MyD88-independent response. In addition, MyD88-deficient mice retain LPS-activated phosphorylation of mitogen-activated protein kinase (MAPK) family members, including ERK1/2, p38 kinase and Jun kinase (JNK), and show LPS-induced activation of NF-κB, although each of these events is slightly delayed[12]. Although it was suggested that a second cytoplasmic TIR-domain protein, known as Mal (also called Tirap), might serve the MyD88-independent pathway[15,16], genetic evidence revealed that Mal and MyD88 act strictly in conjunction with one another[17,18]. The receptor-proximal components of the MyD88-independent signalling pathway have therefore remained uncertain. MyD88-independent signalling might depend on several proteins operating in parallel, or alternatively, might reflect the activity of a single protein or protein complex, carrying signals from TLR4. We have now resolved the molecular basis of MyD88-independent signalling through a forward genetic approach, and herein, we report that this pathway plays a key part in the innate immune response to both viral and bacterial stimuli.

Identification and characterization of the *Lps2* phenotype

In an effort to identify new and essential components of all TLR signalling pathways, we initiated a programme of random germline mutagenesis in mice, using the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU). Both F_1 and F_3 germline mutants were produced on the C57BL/6 background, and males were examined by collecting thioglycolate-elicited peritoneal macrophages under anaesthesia, and testing the integrity of TLR responses *in vitro*. TNF production was measured as an endpoint of signalling in this screen, using the L929 bioassay. LPS, lipid A, peptidoglycan, $PAM_3CysSer(Lys)_4$, resiquimod, dsRNA and CpG-oligonucleotides were used as inducing agents. A total of 2,649 F_1 mice (carrying heterozygous mutations) and 4,584 F_3 mice (carrying homozygous mutations) have so far been screened for new innate immune phenotypes.

Among these animals, a mouse with strong resistance to lipid A and LPS was identified in the F_3 population[19], and was inbred to generate a homozygous mutant stock. In our initial phenotypic characterization of the mutation, dubbed *Lps2*, we found that not only LPS and synthetic lipid A (which signal through TLR4)[1] but also dsRNA (which signals through TLR3)[5] were incapable of activating TNF production in macrophages from homozygous mutants (Fig. 1a–c). LPS-induced macrophage cytotoxicity was also abolished by the mutation (Fig. 1h). The mutation had a far weaker effect on IL-12 and IL-6 production (not shown), and the integrity of signalling through TLR1, -2, -7 and -9 was entirely normal (Fig. 1d–g). Zymosan, which signals through TLR2 and TLR6 heteromers, also produced a normal response (not shown).

The MyD88-dependent pathway, as it proceeds in the course of TLR4 activation, involves recruitment of both MyD88 (ref. 12) and Mal[17,18], and leads to the phosphorylation of ERK1/2, p38 MAP kinase and JNK, and the transient degradation of IκB. These events occurred in LPS-activated macrophages from normal and *Lps2* homozygous mutants, although slightly early ERK1/2 phosphorylation, early dephosphorylation and early regeneration of IκB were consistently evident (Fig. 2a). On the other hand, dsRNA did not stimulate a response in cells from *Lps2* homozygotes (Fig. 2b). The *Lps2* mutation inhibited LPS-induced interferon-β messenger RNA accumulation (Fig. 2c), and type I interferon activity was undetectable in the culture medium of LPS- or dsRNA-activated macrophages from *Lps2* homozygotes (Fig. 2d). LPS-induced NO production as well as inducible NO synthase (iNOS) synthesis, known to be stimulated by type I interferons, were abolished by the mutation (Fig. 2e). LPS- and dsRNA-induced STAT1 phosphorylation was impaired in *Lps2* mutant cells as well, although STAT1 phosphorylation occurred normally in homozygous mutant cells after addition of interferon-β to the culture (Fig. 2f).

All of these effects pointed to proximal blockade of the MyD88-independent pathways serving TLR3 and TLR4. Suspecting a defect upstream of interferon-β, we examined the formation of phosphorylated IRF-3 dimers in LPS- and dsRNA-activated macrophages by western blotting. Heterozygotes for the *Lps2* mutation showed impaired LPS-induced IRF-3 phosphodimer formation (not shown); homozygotes showed no IRF-3 dimerization (Fig. 2g).

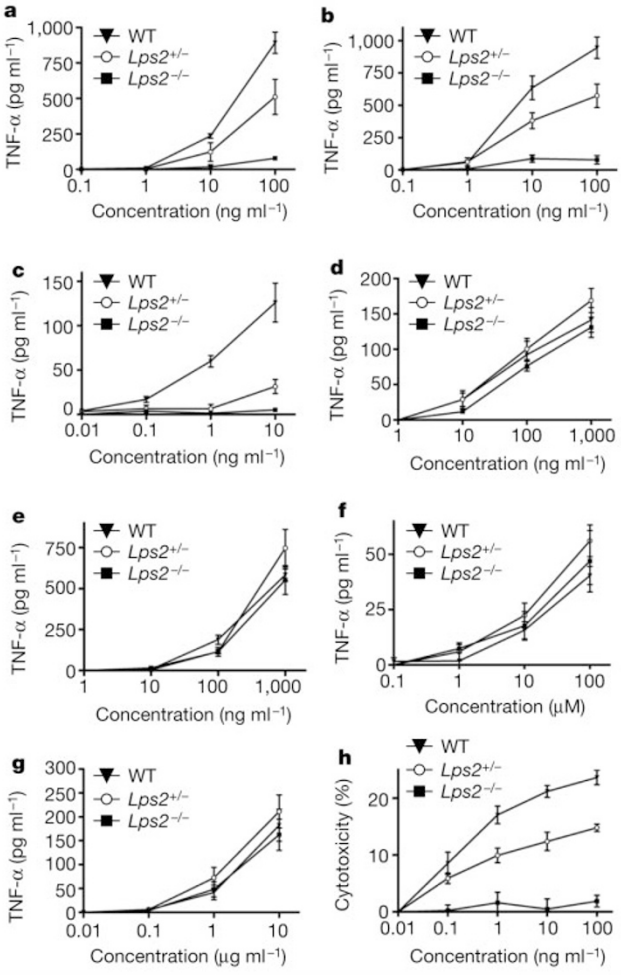

Figure 1 TLR3 and TLR4 specificity of the Lps2 mutation. Mice were injected intraperitoneally with 3% thioglycolate. After 4 days macrophages were isolated and cultured at a density of 5×10^5 cells per well in 96-well plates. Dose–response experiments were performed for each specific inducer. After 4 h of incubation at 37 °C, supernatants were collected and assayed in duplicate for TNF-α activity as described previously[32]. LPS cytotoxicity was evaluated after 24 h of incubation. Values represent means±s.e.m. ($n = 6$ mice). **a**, Lipid A; **b**, LPS; **c**, poly(I:C); **d**, $PAM_3CysSer(Lys)_4$; **e**, resiquimod; **f**, CpG-containing DNA; **g**, peptidoglycan; **h**, LPS-induced cytotoxicity.

Effects of Lps2 homozygosity

Despite the apparent integrity of the MyD88–Mal signalling pathway, known to be required for full expression of endotoxicity[12,17], *Lps2* homozygotes are markedly resistant to the lethal effect of challenge with a 1-mg dose of *Escherichia coli* LPS (Fig. 3a). It should be noted that all of the mutant animals became ill, and some of them died after LPS challenge. Hence, they were visibly less resistant than mice lacking TLR4[1,20]. Nonetheless, it is clear that the MyD88-independent pathway contributes to endotoxicity: a finding consistent with the recent demonstration that interferon-β and its signalling apparatus has an important role in LPS-induced shock[21].

In addition to their faulty response to *E. coli* LPS, *Lps2* homozygotes are compromised in their response to infection with mouse cytomegalovirus (mCMV). Three of eight mutant mice died after challenge with an inoculum (5×10^5 plaque-forming units (PFU)), whereas 30 out of 30 of normal C57BL/6 mice survived, and 1,000-fold higher viral titres were present in the spleens of *Lps2* homozygotes as compared with C57BL/6 controls. BALB/c mice, which fail to eliminate the virus as a result of a mutation in the NK-activating receptor LY49H[22,23], were used as positive controls and developed still higher titres of the virus (Fig. 3b). It remains to be determined whether TLR3 or a signalling protein yet to be identified is of critical importance in these responses, but it is clear that *Lps2* is an essential element of a response pathway required for containment of infection.

As the *Lps2* mutation abolished LPS- and dsRNA-induced type I interferon production by macrophages *in vitro*, we examined type I interferon levels in the serum of mCMV-infected mice and in culture medium collected from mCMV-infected macrophages. Robust production of type I interferon was observed in the serum of normal mice, but no type I interferon was detected in the serum of *Lps2* mutant homozygotes (Fig. 3c). The response of macrophage cultures to infection by *Vaccinia* virus was demonstrably impaired, in that macrophage monolayers from *Lps2* homozygotes supported the replication of *Vaccinia* to a higher titre than did macrophages from normal mice (Fig. 3d). Hence, the antiviral effect conferred by the normal *Lps2* product is very broad with regard to the virus species, correlated with type I interferon production, and is at least largely independent of an adaptive immune response. Moreover, the normal *Lps2* product is required for a type I interferon response to viral infection *in vivo*.

Genetic mapping and sequencing reveals the identity of *Lps2*

Lps2 was mapped meiotically by out-crossing homozygotes to C3H/HeN mice and backcrossing the progeny to the mutant stock. Using a panel of 59 genome-wide microsatellite markers applied to 26 meioses, *Lps2* was first confined to an interval on mouse chromosome 17 (Supplementary IS-1), and then, on a total of 1,567 meioses, to a region delimited by microsatellite markers D17Mit87 and D17Mit20. Within this region, eleven additional microsatellite length polymorphisms were identified in our laboratory (Supplementary IS-4). A total of seven crossovers were observed between D17Mit87 and D17Mit20, and these crossovers were resolved by haplotype analysis with respect to three of the internal markers, permitting optimal confinement of the *Lps2* mutation (Supplementary IS-2). A single crossover was observed between the proximal marker Lps2_7_28 and *Lps2*. A single crossover was also observed between *Lps2* and the distal marker Lps2_7_34. The length of the critical region, thus defined, was 216 kilobases (kb). The critical region was amplified in 63 overlapping 4-kb fragments using template amplified from an *Lps2*

homozygous mutant and from a normal C57BL/6 mouse, and sequenced in entirety on both strands with a panel of 865 internal primers. Only a single mutation was present in the critical region of the *Lps2* strain, and it was identified within one of eight putative genes that reside within the region (Supplementary IS-3a). This candidate, although fragmentary and unannotated in the Ensembl database, was considered exceptionally promising because it encodes a TIR-domain-containing protein. Two reports have previously suggested that this protein, elsewhere called Trif[24] or Ticam-1 (ref. 25), might indeed have a role in TLR signalling, and particularly in the activation of interferon-β gene expression. However, no consensus was established as to the TLRs that depend on it; one report suggesting association with numerous TLRs and MyD88 (ref. 24), the other an exclusive association with TLR3 (ref. 25).

The length and content of the murine *Trif* gene was deduced by analysis of the numerous expressed sequence tags that have been derived from it, and confirmed by rapid amplification of cloned ends (RACE) of macrophage-derived complementary DNA. In normal C57BL/6 mice, the gene encodes a protein 732 residues in

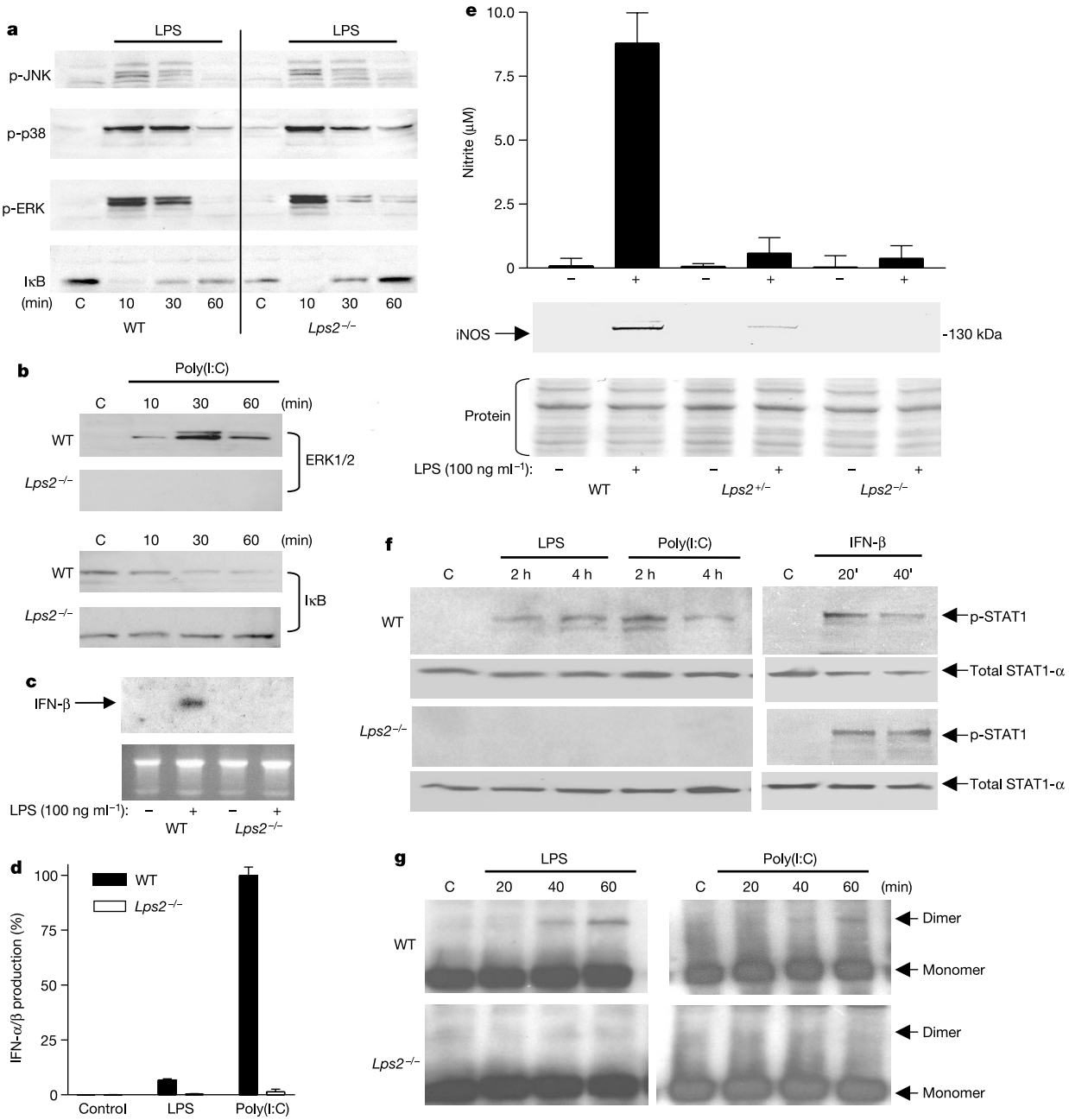

Figure 2 *Lps2* is required for MyD88-independent TLR3 and TLR4 signalling in macrophages. **a**, **b**, Western blot analysis of phospho-MAPKs and IκB after macrophage stimulation with LPS (**a**) or poly(I:C) (**b**). **c**, Northern blot analysis after 1 h incubation with or without LPS. **d**, Type I interferon activity measured after 2 h incubation with LPS (100 ng ml^{-1}) or poly(I:C) (10 μg ml^{-1}). Error bars indicate s.e.m.; $n = 2$ mice. **e**, iNOS (western blot) and NO (Griess assay) were measured after 24 h incubation with or without LPS. Protein was stained with Ponceau S. Error bars indicate s.e.m.; $n = 6$. **f**, **g**, Western blot analysis of STAT1 (**f**) and IRF-3 phosphodimer formation (**g**); LPS, 100 ng ml^{-1}; poly(I:C), 10 μg ml^{-1}.

length. In *Trif*^{Lps2} homozygotes, the Trif protein is modified by a single base-pair deletion, predicted to remove 24 amino acids from the carboxy terminus of the protein, replacing them with an unrelated sequence 11 amino acids in length (Supplementary IS-3b, c). Because this was the sole mutation present in the *Lps2* critical region, as the mutation markedly alters the *Trif* coding sequence, and as the mutant phenotype is very much consistent with modification of a TIR-domain-containing protein, we concluded that the *Lps2* phenotype results from the observed *Trif* mutation. This conclusion was supported by phenotypic rescue experiments in which macrophages were transfected with an expression vector encoding normal Trif. Transfected cells, identified by gating on fluorescence created by co-transfection with a yellow fluorescent protein (YFP) expression vector, were capable of producing TNF in response to dsRNA (Fig. 4). Moreover, the findings suggest that the mutant product may exert a dominant inhibitory effect.

Notably, the 19 C-terminal residues of the mouse Trif protein are not represented in the human homologue of the protein, which is nonetheless functional in our own transfection system and in those reported elsewhere[24,25]. Moreover, a large C-terminal deletion of the human protein reportedly failed to impede Trif-mediated signal transduction when expressed in mammalian cells; only a combined amino-terminal/C-terminal deletion (ΔNΔC) had dominant inhibitory properties[24]. These considerations may indicate that the 11-residue aberrant C-terminal sequence created by the *Lps2* frameshift error causes instability or inactivation of the mutant protein. The co-dominant character of the *Trif*^{Lps2} allele would in this context probably be explained on the basis of a quaternary structural effect. The possibility that the locus is also haploinsufficient cannot, as yet, be excluded. A null allele of *Trif* (for example, deletion of the locus) might conceivably yield a distinguishable phenotype, more consistent with either of the previous *in vitro* studies[24,25], but this remains to be established.

A tBLASTn search using the Trif protein as a query retrieves another gene encoding a TIR domain polypeptide, localized to mouse chromosome 18 (46.7 megabases from the centromere; ENSMUST00000036100). This, the closest homologue of Trif, is also represented in the human genome, and seems to be a cytoplasmic adaptor protein similar to Trif itself. As yet, no function has been ascribed to this homologue. We suggest that it may be involved in TIR-related signal transduction, and consider it possible that it also has an essential role in Trif-independent signalling, detailed below.

The bifid nature of the LPS signalling pathway

As described above, TLR3 and TLR4 share a common signalling intermediate, Trif, which is absolutely required for IRF-3 phosphodimer formation occurring in response to LPS, dsRNA or authentic viral infections, and all subsequent events related to type I interferon production and action. Particularly with regard to LPS signalling, however, it should be noted that some of the events that occur in MyD88-deficient mice, such as the tardive phosphorylation of ERK1/2, p38, JNK, and activation of NF-κB, also occur in homozygous *Trif*^{Lps2} mutants. Hence, two possibilities present themselves. On the one hand, Trif and the MyD88–Mal complex might each be capable of initiating these 'shared' endpoints of TLR signalling independently. In the most parsimonious model, all LPS signals might traverse two (and only two) 'branches', one relatively rapid and dependent on MyD88 and Mal, the other slightly slower and dependent on Trif. On the other hand, an unknown number of signalling adaptors might be responsible for MAPK family and NF-κB activation independent of both Trif and the MyD88–Mal complex. In this alternative, the MyD88-independent pathway would not be unitary, but would comprise at least two and perhaps several pathways, each arising independently from the LPS receptor.

To make a clear distinction between these alternatives, we sought to determine whether any signals at all emanate from the activated LPS receptor in *Trif*$^{Lps2/Lps2}$; *MyD88*$^{-/-}$ mice. To produce compound homozygous mutants, we crossed *MyD88*$^{-/-}$ mice (a gift of

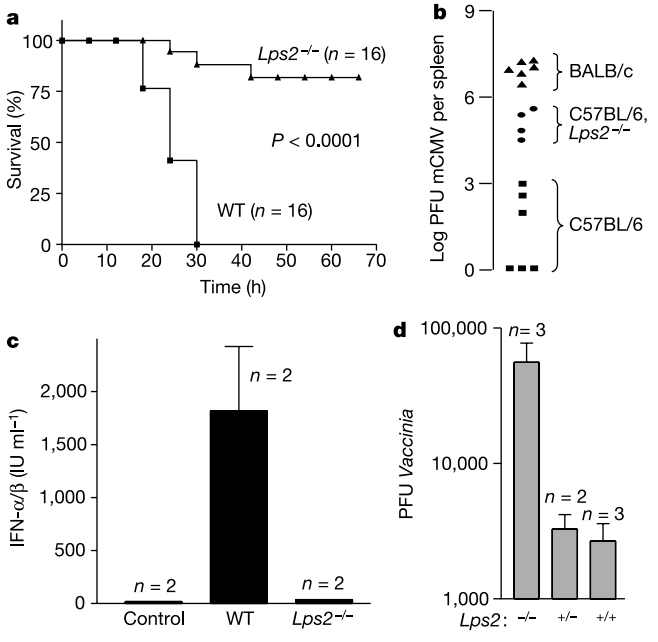

Figure 3 Responses to bacterial and viral stimuli in *Lps2* homozygotes. **a**, Mice were injected intraperitoneally with 1 mg of *E. coli* LPS and monitored for survival. **b**, Mouse cytomegalovirus (mCMV) infection. Each mouse received 5×10^5 PFU intraperitoneally. Spleens were assayed for infectious virus by plaque assay using NIH 3T3 cells at 5 days after inoculation. **c**, Type I interferon activity measured in the serum of mCMV-infected mice[31], 1.5 days after inoculation. Error bars indicate s.e.m. **d**, 4×10^6 peritoneal macrophages were infected with 4×10^5 PFU of *Vaccinia* virus (titred on HeLa cells); assay of viral product was performed at 3 days after inoculation. *n* indicates the number of mice; error bars indicate 95% confidence limits.

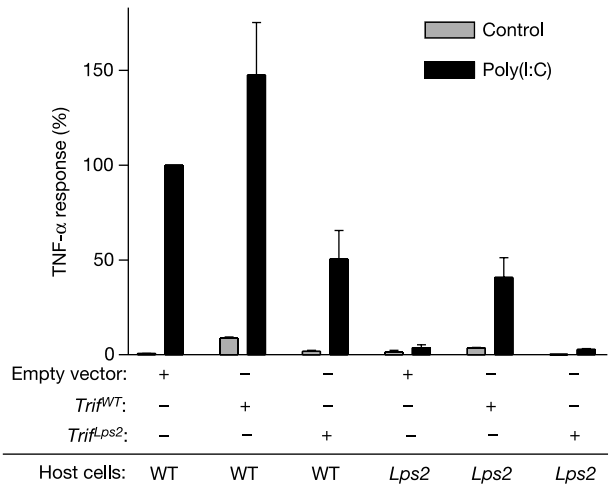

Figure 4 Rescue of poly(I:C) responsiveness in *Lps2* homozygous mutant macrophages by transfection with normal cDNA. Relative quantity of TNF in macrophages from normal and mutant mice, transfected with constructs shown, with or without poly(I:C) induction, 10 μg ml^{-1} for 4 h. All values are normalized with respect to the quantity of TNF expressed by induced wild-type cells transfected with empty vector (100%). Error bars indicate s.e.m. of three independent experiments. WT, C57BL/6 macrophages; *Lps2*, macrophages from homozygous mutant mice.

S. Akira) to *Trif*^{Lps2} homozygotes, and intercrossed the offspring. Among 88 F_2 animals examined, three *Trif*$^{Lps2/Lps2}$;*MyD88*$^{-/-}$ animals were identified when genotyped at three months of age. Peritoneal macrophages obtained from these animals were examined for phosphorylation of MAPK family members and transient degradation of IκB (Fig. 5).

Single-locus mutants both showed normal levels of ERK1/2 phosphorylation and transient degradation of IκB. As previously described[12], *MyD88* deletion causes a slight delay in these events, but does not abrogate them. Homozygosity for the *Trif*^{Lps2} allele resulted in slightly earlier phosphorylation by comparison, with a concomitant early decline in the signal. Compound homozygotes showed no ERK1/2 phosphorylation responses (Fig. 5a), nor transient degradation of IκB (Fig. 5b). Precisely the same pattern of response was observed for p38 kinase and JNK (not shown). Although more extensive studies may ultimately reveal endpoints of LPS signalling that are as yet unknown, it seems for the present that MyD88–Mal signalling and Trif signalling define separate rami of a bifurcated pathway responsible for all of the signals that emanate from the LPS receptor. As it is well known that all LPS signals are silenced by the P732H mutation of TLR4 (ref. 1), which does not seem to alter receptor shape[26] and is consistent with the surface expression of TLR4 (ref. 27), it may be inferred that the separate adaptors both engage the same domain of the receptor, and are probably in competition with one another.

Both branches of the LPS signalling pathway are required for the full expression of LPS toxicity *in vivo*, and it is clear that the two branches are superadditive, insofar as integrity of either locus yields only about 10% of the amount of TNF produced by wild-type cells (Fig. 5c). Synergy between the MyD88–Mal pathway and the Trif pathway might occur distally as well, through the cooperative elicitation of cytokines and other effector proteins, many of which are transcriptionally activated by both STAT1 and NF-κB. Insofar as LPS-induced macrophage apoptosis is abolished by the *Trif*^{Lps2} mutation (Fig. 1h), we conclude that pro-apoptotic signals emanate exclusively from the Trif pathway. As a double mutation is required to prevent MAPK phosphorylation and IκB degradation, we conclude that both Trif and MyD88–Mal are independently capable of initiating these events, the former more slowly than the latter. Although the consequences of MyD88–Mal signalling are temporally distinguishable from those of the Trif pathway, we have not, as yet, identified a qualitatively unique effect of MyD88–Mal signalling induced by LPS. It is unclear from our own data whether Trif directly engages IRF-3 and/or whether it engages an essential kinase that does so[28].

Characteristics of a Trif-independent signalling pathway

Just as it is possible to speak of MyD88-dependent and MyD88-independent signalling, it is also possible to speak of Trif-dependent and Trif-independent signalling, as the mutation that we have observed does not fully abolish LPS signal transduction, although it does fully abolish dsRNA signal transduction. Notably, Trif-dependent and Trif-independent signalling pathways reside within separate cell populations (Fig. 6a, b).

All CD11b-positive, F4/80-positive macrophages in a thioglycolate-elicited peritoneal exudate from wild-type mice respond to LPS (Fig. 6a, top), whereas only a fraction of the cells respond to dsRNA (Fig. 6b, top) by producing TNF. Macrophages from *Trif*$^{Lps2/Lps2}$ mice are nearly unresponsive to dsRNA (Fig. 6b, bottom); however,

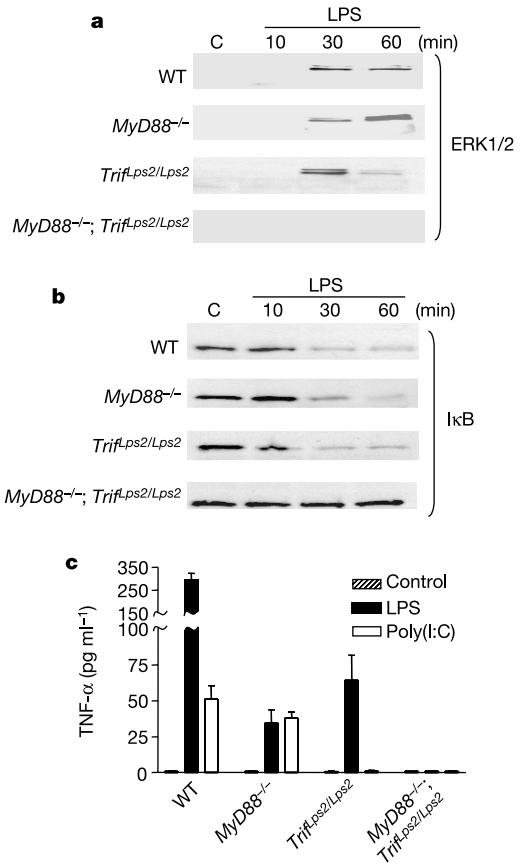

Figure 5 Single and compound homozygosity for *MyD88* and *Trif* mutations: evidence for a bifid LPS response pathway and a unitary dsRNA response pathway. **a**–**c**, Macrophages were left unstimulated or activated with LPS (10 μg ml^{-1}) and analysed for phosphorylation of ERK1/2 (**a**), IκB (**b**), or TNF-α secretion measured by bioassay (**c**). Each bar represents 2–4 mice; error bars indicate s.e.m.

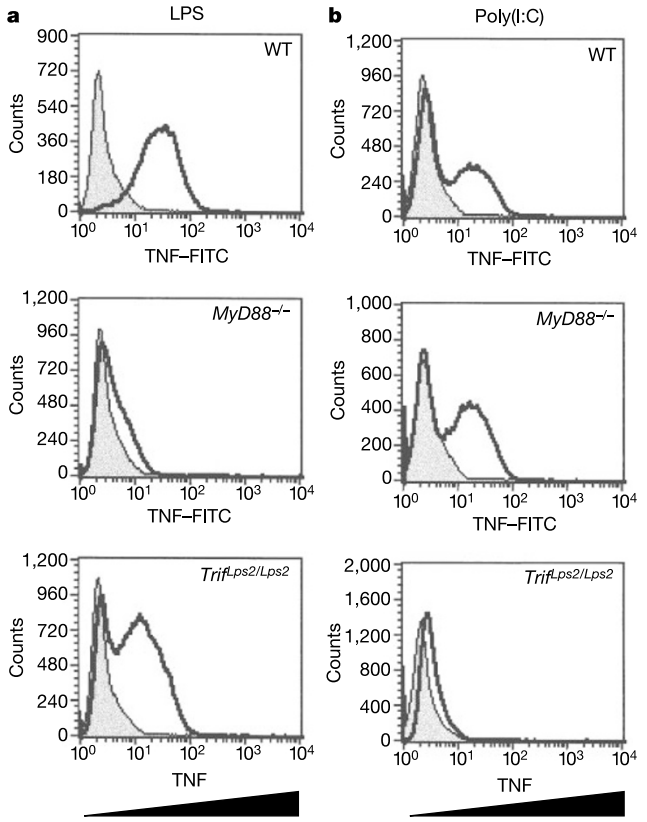

Figure 6 Evidence for functionally distinct populations of macrophages. Distribution of TNF-α accumulation in cells left unstimulated (shaded) or stimulated (unshaded) for 4 h with LPS (100 ng ml^{-1}) or poly(I:C) (10 μg ml^{-1}).

the same genotype yields two types of response to LPS: some cells show a relatively weak response to LPS (low TNF production), and other cells show no response at all (Fig. 6a, bottom). MyD88 deficiency yields a single population of cells that show a uniformly low response to LPS (Fig. 6a, middle) but a dsRNA response that is indistinguishable from the wild type (Fig. 6b, middle), consistent with the results of culture medium assays (Fig. 5c).

Therefore, with regard to the LPS response, macrophages are of two types: Trif-dependent and Trif-independent. Some $Trif^{Lps2/Lps2}$ macrophages lack LPS responsiveness, whereas all $Trif^{Lps2/Lps2}$ macrophages lack dsRNA responsiveness. Because MyD88 appears to be expressed by all macrophages (Fig. 6a, middle), it would follow that a separate adaptor protein ('adaptor X') may permit the diminished LPS responses that occur in Trif-independent cells. As no TNF is induced by LPS in $Trif^{Lps2/Lps2}$;$MyD88^{-/-}$ mutant macrophages (Fig. 5c), we posit that adaptor X is capable of functioning only in cells that co-express MyD88; that is, similar to Mal, adaptor X is MyD88-dependent. Where dsRNA responses are concerned, adaptor X cannot substitute for Trif.

The closest homologue of *Trif* that we have earlier noted to be present in the mouse genome stands as a candidate to fulfil the role of adaptor X, active in the Trif-independent cell population but not in the Trif-dependent cell population. Trif-independent cells are probably capable of specialized responses, by virtue of the fact that they express a unique adaptor molecule.

Receptor diversity meets adaptor degeneracy

We suggest that clinical similarity between certain viral and bacterial diseases is largely explained by the sharing of TIR domain transducers. These transducers serve innate immune responses to microbes that are structurally unrelated, and therefore recognized by different receptors. Trif is one such transducer, and its mutational modification impairs responses to both viral infections and bacterial LPS. Notwithstanding its ability to detect diverse pathogens, the TLR system has a limited repertoire of responses. To some extent, however, degeneracy may be offset by afferent functional specialization among innate immune cells. □

Methods

Innate immune activators, probes and antibodies

Escherichia coli RE595 LPS was a gift of C. Galanos. Synthetic *E. coli* lipid A was produced in the laboratory of S. Kusumoto. dsRNA (poly(I:C)) was purchased from Amersham Pharmacia Biotech. $Pam_3CysSer(Lys)_4$ was obtained from EMC microcollections GmbH and peptidoglycan was purchased from Fluka. Phosphorothioate-stabilized CpG oligodeoxynucleotide (ODN) 5′-TCCATGACGTTCCTGATGCT-3′ was obtained from Integrated DNA Technologies. Phosphospecific antibodies for ERKs, p38 and JNK were obtained from New England Biolabs, and antibodies against IκB and IRF-3 were purchased from Santa Cruz Biotechnology. Rabbit anti-mouse-iNOS antibodies were obtained from Cayman Chemical. STAT1 [pY701] phosphospecific antibodies and antibodies against total STAT1α were obtained from Biosource International. A MuIL-12p40 ELISA and rMuTNF-α were purchased from Pierce Endogen. rMuIFN-β was obtained from R&D systems. The 340-bp IFN-β hybridization probe was amplified from genomic DNA using a forward primer (5′-CTTCTCCACCACAGCCCTCTCCATC-3′) and a reverse primer (5′-GAGCAGTTGAGGACATCTCCCACGTC-3′).

Western blotting

Western analysis of JNK, p38, STAT and ERK1/2 phosphorylation, and degradation of IκB was performed as previously described[29]. IRF-3 phosphodimer detection was performed as described in ref. 30.

Type I interferon assay

Type I interferon assay was performed according to the method of ref. 31.

Macrophage transfection and FACS analysis

Thioglycolate-elicited peritoneal macrophages were transfected for 48 h with YFP vector alone, or mixed at a 1:3 molar ratio with either *Trif* or $Trif^{Lps2/Lps2}$ cDNA expressed under the influence of a CMV promoter. Transfection was performed using Geneporter transfection reagent (Gene Therapy Systems). Cells were induced in the presence of brefeldin A with poly(I:C) ($10\,\mu g\,ml^{-1}$), or left uninduced.

Germline mutagenesis

We obtained ENU from Sigma and used it as described[19].

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Correspondence and requests for materials should be addressed to B.B. (bruce@scripps.edu).