non-adherent cells were plated (1×10^5 ml^{-1}) and cultured for 6 days. Unactivated macrophages were stimulated with *L. monocytogenes* using a multiplicity of infection of 20, whereas macrophages activated with IFN-γ (400 U ml^{-1}) for 16 h were stimulated with LPS. Nuclear extracts (3 μg) were incubated with a ^{32}P-labelled oligonucleotide probe derived from the major histocompatibility complex (MHC) class II NF-κB consensus-binding site, and analysed by EMSA.

Transient transfection and luciferase analysis

Human embryonic kidney (293T) cells were plated in 6-well plates at 2.5×10^5 cells per well. Cells were stimulated by transfection with plasmid pCMV (cytomegalovirus) human NOD1 (10 ng) or pCMV murine CD4-TLR4 (10 ng). Stimulated cells were inhibited by co-transfection with pCMV human RIP2-DN (1–434), pcDNA3 murine MyD88-DN, pCiNeo murine IRAK-DN, or pCMV IκB-DA, as indicated (in ng). All transfections were performed with a luciferase reporter plasmid containing two κB binding sites (20 ng) and pCMV-LacZ (40 ng). Luciferase activity was measured and normalized to beta-galactosidase activity.

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Competing interests statement

The authors declare that they have no competing financial interests.

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RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems

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The immune system consists of two evolutionarily different but closely related responses, innate immunity and adaptive immunity. Each of these responses has characteristic receptors—Toll-like receptors (TLRs) for innate immunity and antigen-specific receptors for adaptive immunity. Here we show that the caspase recruitment domain (CARD)-containing serine/threonine kinase Rip2 (also known as RICK, CARDIAK, CCK and Ripk2)[1–4] transduces signals from receptors of both immune responses. Rip2 was recruited to TLR2 signalling complexes after ligand stimulation. Moreover, cytokine production in Rip2-deficient cells was reduced on stimulation of TLRs with lipopolysaccharide, peptidoglycan and double-stranded RNA, but not with bacterial DNA, indicating that Rip2 is downstream of TLR2/3/4 but not TLR9. Rip2-deficient cells were also hyporesponsive to signalling through interleukin (IL)-1 and IL-18 receptors, and deficient for signalling through Nod proteins—molecules also implicated in the innate immune response. Furthermore, Rip2-deficient T cells showed severely reduced NF-κB activation, IL-2 production and proliferation on T-cell-receptor (TCR) engagement, and impaired differentiation to T-helper subtype 1 (T_H1) cells, indicating that Rip2 is required for optimal TCR signalling and T-cell differentiation. Rip2 is therefore a signal transducer and integrator of signals for both the innate and adaptive immune systems.

Rip2 is a serine/threonine kinase[1–4] that carries a CARD at its carboxy terminus, and shares sequence similarity with a serine/threonine kinase, Rip, which is essential for NF-κB activation through the tumour-necrosis factor (TNF) receptor[5]. *In vitro* studies have shown that Rip2 can associate with a variety of other CARD-containing molecules through CARD–CARD interactions[1–3]. Moreover, overexpression of Rip2 causes activation of NF-κB and Jun amino-terminal kinase (JNK)[1–4]. NF-κB activation by Rip2 is inhibited by dominant negative TRAF6 (ref. 4), a signalling molecule that is downstream of TLRs. Furthermore, we found that expression of Rip2 was induced in macrophages on stimulation with lipopolysaccharide (LPS) (Fig. 1a, b). These observations led us to consider the possibility that Rip2 is involved in signalling in the innate immune system. To assess the physiological role of Rip2 in signalling in the innate immune system, we generated Rip2-deficient mice by homologous recombination of embryonic stem cells. A gene-targeting construct was generated to replace the two exons coding for murine *Rip2* with a neomycin-resistance gene (*neo*^{r}) (Fig. 1c). Homologous recombination in embryonic stem cells was confirmed by Southern blot analysis (Fig. 1d), and the absence of Rip2 expression in homozygous animals was confirmed by western blot (Fig. 1e). Rip2-deficient mice were born in the expected mendelian ratio, and showed no gross developmental abnormalities and no abnormal composition of lymphocytes as determined by flow cytometry (data not shown).

TLRs can recognize specific pathogen-associated molecular patterns (PAMPs) such as LPS[6], lipoteichoic acid (LTA)[7], peptidoglycan (PGN)[7], CpG-containing DNA[8], or double-stranded RNA[9]. To test whether Rip2 is involved in TLR signalling, macrophages of *Rip2*$^{-/-}$ mice were stimulated with various PAMPs, and cytokine/chemokine production was assessed by enzyme-linked immunosorbent assay (ELISA). Production of the inflammatory cytokines IL-6 and TNF-α, and the chemokine IP10, was severely reduced in macrophages of *Rip2*$^{-/-}$ mice on stimulation with LPS and LTA (both of which are ligands for TLR4), or PGN (which is a ligand for TLR2) (Fig. 2a). There was no defect in cytokine/chemokine production after stimulation with CpG DNA (a ligand for TLR9), indicating that Rip2 is involved in TLR4 and TLR2 but not TLR9 signalling. Production of IL-6 is also reduced in *Rip2*$^{-/-}$ embryonic fibroblasts on stimulation with double-stranded RNA, poly(IC) (a ligand for TLR3) and LPS in a dose- and time-dependent manner (Fig. 2c). These results indicate that Rip2 is required for normal levels of signalling through some, but not all, TLRs. To assess the response to a live pathogen, macrophages of *Rip2*$^{-/-}$ mice were infected with *Listeria monocytogenes*, and the levels of IL-6 and TNF-α were measured by ELISA. Macrophages of *Rip2*$^{-/-}$ mice were compromised in their ability to produce these cytokines, further supporting the involvement of this kinase in the innate immune response (Fig. 2b and data not shown). To examine the mechanism of activation of Rip2 in macrophages infected with *L. monocytogenes*, in particular to determine whether stimulation of the innate immune response by *Listeria* occurs at the cell surface or intracellularly, cytochalasin D was added to cell cultures at various concentrations, as it blocks *Listeria* internalization[10]. IL-6 production in response to *Listeria* infection was not altered even at high concentrations of cytochalasin D, and in all cases was reduced in *Rip2*$^{-/-}$ cells (Fig. 2b). Thus, attachment of bacteria to the cell surface is sufficient to activate macrophages, and the reduced cytokine production in *Rip2*$^{-/-}$ cells is due to defective signalling from cell-surface receptors. *In vivo* response to LPS by Rip2-deficient mice was assessed by endotoxin shock experiments using intraperitoneal injection of LPS. Rip2-deficient mice were more resistant to LPS than wild-type mice (Fig. 2d), suggesting the importance of this molecule in LPS response *in vivo*.

Mature IL-1β can be produced through cleavage of pro-IL-1β by active caspase-1. Previous *in vitro* studies have suggested that Rip2 binds and activates pro-caspase-1, thereby generating IL-1β[3,11]. Notably, macrophages of *Rip2*$^{-/-}$ mice exhibited no reduction in IL-1β production when cells were stimulated with LPS, CpG DNA and peptidoglycan for 3 h (Supplementary Information), suggesting that Rip2 is not involved in caspase-1 activation downstream of TLRs, or alternatively that there is another redundant mechanism.

TLR signalling requires the formation of multiprotein signalling complexes, which include the serine/threonine kinase IRAK and the adapter molecules MyD88 and TRAF6, resulting in the activation of

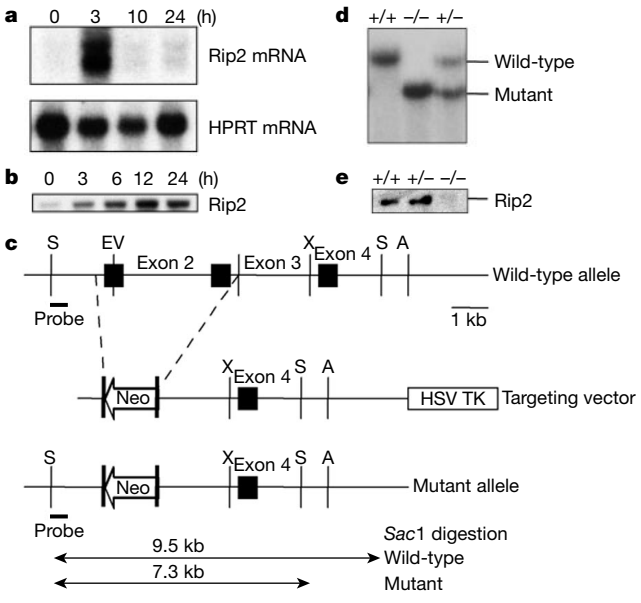

Figure 1 Expression of Rip2 in macrophages and targeted disruption of the mouse *Rip2* gene. **a**, **b**, Northern and western blot analysis for Rip2 expression in macrophages stimulated with 10 ng ml^{-1} LPS for the indicated times. HPRT, hypoxanthine guanine phosphoribosyltransferase. **c**, Diagram of the *Rip2* locus, the targeting vector and the targeted allele. Filled boxes denote exons. Restriction enzyme sites are indicated (S, *Sac*I; EV, *Eco*RV; X, *Xba*I; A, *Apa*I). TK, thymidine kinase. **d**, Southern blot analysis of *Sac*I-digested genomic DNA identifies mice of all three expected genotypes. **e**, Western blot analysis of thymocytes showing the absence of Rip2 protein in homozygous mice.

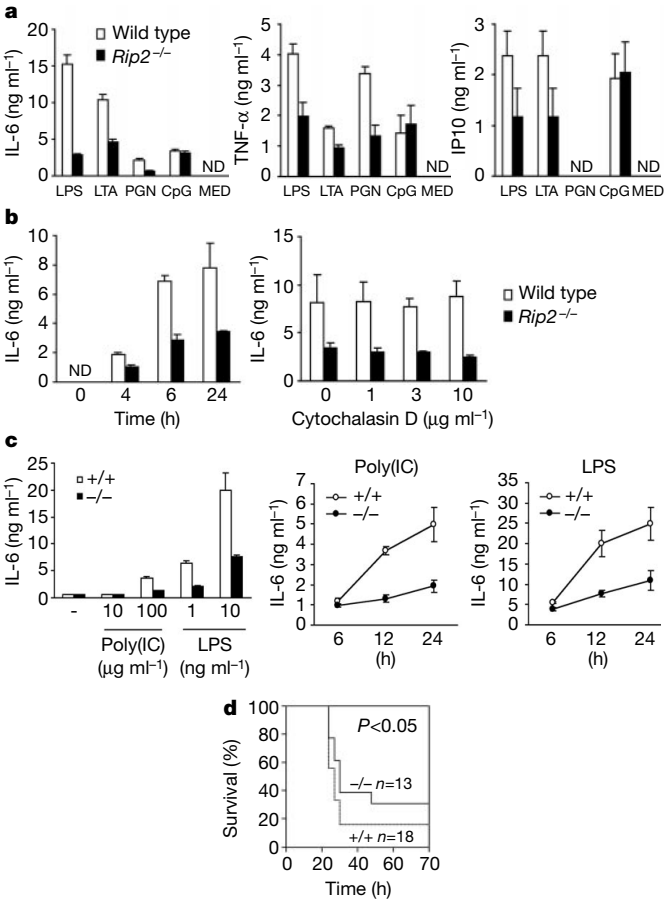

Figure 2 Impaired TLR responses in Rip2-deficient cells. **a**, Production of IL-6, TNF-α and IP10 by *Rip2*$^{-/-}$ macrophages derived from bone marrow, stimulated with LPS (10 ng ml^{-1}), LTA (10 μg ml^{-1}), PGN (10 μg ml^{-1}), CpG oligonucleotide DNA (CpG, 10 μM), and medium alone (MED) for 6 h. ND, not detected. **b**, Production of IL-6 by *Rip2*$^{-/-}$ macrophages infected with *L. monocytogenes* and cultured for the indicated period (left), or cultured for 6 h in the presence of the indicated concentrations of cytochalasin D (right). **c**, Production of IL-6 by *Rip2*$^{-/-}$ fibroblasts stimulated with poly(IC) or LPS with indicated dose for 24 h (left), or stimulated with 100 μg ml^{-1} poly(IC) (middle) or 10 ng ml^{-1} LPS (right) for indicated periods. **d**, Survival curve of wild-type and *Rip2*$^{-/-}$ mice after endotoxin shock. LPS (16.7 mg kg^{-1} body weight) was injected intraperitoneally into wild-type and *Rip2*$^{-/-}$ mice. Viability was determined every 3 h for the first 36 h, followed by every 12 h. There was no incremental death after 80 h. *P*-values were determined by the Mantel–Cox test. Experiments were performed with triplicates and were repeated 6 (**a**, IL-6), 3 (**a,** TNF-α/IP10) and 2 (**b**, **c**) times, with equivalent results.

NF-κB and the mitogen-activated protein (MAP) kinases JNK, p38 and ERK1/2 (refs 12, 13). We therefore studied activation of these molecules in macrophages of *Rip2*$^{-/-}$ mice by examining their phosphorylation state. LPS-stimulated *Rip2*$^{-/-}$ macrophages showed reduced phosphorylation levels of p38, IκBα, ERK and JNK, and reduced degradation of IκBα (Fig. 3a), indicating altered signalling downstream from TLR4. This reduced signalling was not due to changes in the expression levels of MyD88, IRAK or TRAF6, as western blot analysis for these proteins showed no difference between wild-type and *Rip2*$^{-/-}$ macrophages (data not shown). Furthermore, we studied whether Rip2 is recruited to TLR signalling complexes. 293T cells stably transfected with Flag-tagged TLR2 and CD14 (Flag-TLR2/CD14 293T cells) were stimulated with PGN, and association of TLR2 and endogenous Rip2 was examined by immunoprecipitation using an anti-Flag monoclonal antibody. Association of Rip2 with TLR2 was induced by PGN and peaked at 10 min after stimulation; the amount of associated Rip2 decreased after 15 min, indicating that the recruitment of Rip2 to TLR signalling complex is transient after TLR activation (Fig. 3b).

In addition to TLRs, increasing evidence implicates another family of proteins in innate immune responses. The cytoplasmic proteins collectively termed Nod, are characterized by the presence of three motifs: a CARD, a nucleotide-binding domain (NBD) and a leucine-rich repeat (LRR) region. These proteins have homology to the NBD-LRR-like disease resistant proteins in plants[14–16]. An increasing number of members of this family have been identified (Nod1/CARD4, Nod2, DEFCAP/NAC, CARD12/Ipaf/CLAN), and by analogy to the plant molecules these data imply that, like the TLR family, Nod proteins are a diverse family of molecules designed to detect pathogens in intracellular compartments—the LRR region of members of both families probably confers pathogen specificity[17,18]. In fact, Nod1 is activated on infection of *Shigella flexneri* in epithelial cells[19], and one NBD-LRR protein, NAIP, determines susceptibility to *Legionella pneumophila* infection[20]. Also, it has been demonstrated that Nod2 is mutated in patients susceptible to Crohn's disease and Blau syndrome[18,21,22]. *In vitro* studies have revealed that Nod1 and Nod2 bind to Rip2 by means of a CARD–CARD interaction[15,16], suggesting that Rip2 may be involved in signalling downstream of the Nod family proteins. To test this, we generated Rip2-deficient embryonic fibroblasts and co-transfected them with both Nod expression vectors and a NF-κB reporter construct. NF-κB activation by Nod1/Nod2 expression was completely abolished in fibroblasts of *RIP2*$^{-/-}$ mice, and complementation of *Rip2*$^{-/-}$ fibroblasts with a Rip2 expression vector restored these defects (Fig. 4). These results indicate that Rip2 is essential for NF-κB activation by both Nod1 and Nod2. Together, these results indicate that Rip2 is essential for signalling through both protein families of the innate immune system, that is TLRs and Nod.

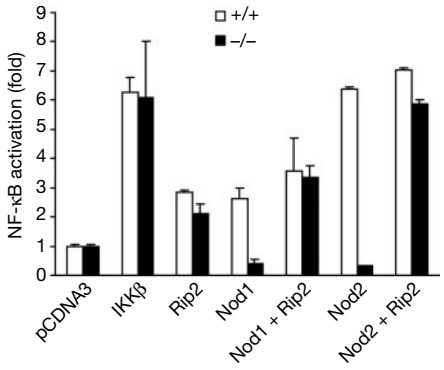

Figure 4 Rip2 is essential for activation of NF-κB by Nod1 and Nod2. Embryonic fibroblasts from wild-type and Rip2-deficient mice were co-transfected with pcDNA3, pcDNA3-IKKβ, pcDNA3-Nod1ΔLRR, pcDNA3-Nod2 or pcDNA3-Rip2, pEF-BOS-β-gal and pBVI-Luc reporter plasmids. The experiments were repeated at least three times with equivalent results.

IL-1 and IL-18 receptors both have a TIR (Toll/IL1-receptor) domain within the cytoplasmic tail, and therefore belong to the same multigene family as the TLRs. We therefore tested whether responses to IL-1 or IL-18 were altered in cells of *Rip2*$^{-/-}$ mice. Under certain conditions, IL-1β is a potent co-stimulant to T cells for their growth. We stimulated thymocytes with IL-1β, together with IL-2 or a low dose of Concanavalin A (Con A). Thymocytes of *Rip2*$^{-/-}$ mice showed reduced proliferation upon IL-1β stimulation in both IL-2 and Con A co-stimulation (Fig. 5a). Embryonic fibroblasts produce IL-6 upon stimulation with the cytokines IL-1β or TNF-α. We found that embryonic fibroblasts of *Rip2*$^{-/-}$ mice were significantly impaired in their ability to produce IL-6 when stimulated with IL-1β, but not when stimulated with TNF-α, suggesting that Rip2 is involved in IL-1- but not TNF-α-receptor signalling (Fig. 5b).

We next investigated the IL-18 response in cells of *Rip2*$^{-/-}$ mice. IFN-γ production by natural killer (NK) cells upon IL-18 stimulation was assessed using splenocytes of *Rip2*$^{-/-}$ mice. IFN-γ production was severely reduced in *Rip2*$^{-/-}$ cells by stimulation with IL-18 (Fig. 5c). Surprisingly, IFN-γ production upon stimulation with IL-12 was also reduced. Co-stimulation of *Rip2*$^{-/-}$ cells with IL-18 and Il-12 also resulted in reduced production of IFN-γ

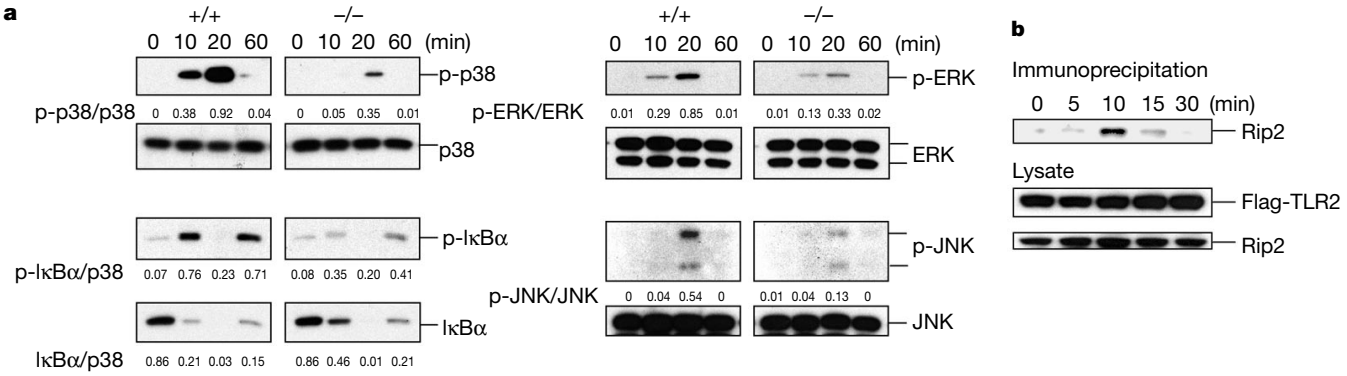

Figure 3 Rip2 is required for optimal TLR signalling. **a**, Rip2 is upstream of multiple signalling pathways, including NF-κB, JNK, p38 and ERK. Macrophages derived from bone marrow of wild-type and Rip2-deficient mice were stimulated with LPS (10 ng ml^{-1}) for indicated periods. Cell lysates were prepared and blotted with the indicated antibodies. Ratios of phosphorylated and unphosphorylated proteins quantified by an image-analyser are shown. These data are the mean of three independent experiments. **b**, Transient recruitment of Rip2 to TLR signalling complexes. Flag-TLR2/CD14 293T cells were stimulated with 10 μg ml^{-1} PGN for the indicated times. Cell lysates were immunoprecipitated with anti-Flag monoclonal antibody and blotted with anti-Rip2 antibody.

(Fig. 5c). Furthermore, we examined IL-18 and IL-12 response using differentiated effector $CD4^+$ T_H1 cells. Similar to NK cells, effector T_H1 cells make copious amounts of IFN-γ when stimulated with IL-18 and IL-12 in the absence of T-cell receptor (TCR) stimulation[23]. IFN-γ production by *Rip2*$^{-/-}$ T_H1 cells stimulated with IL-18, IL-12 or a combination of IL-18 and IL-12, was severely perturbed (Fig. 5d). These results suggest that Rip2 is involved in signalling downstream of the TLR/IL-1 receptor family, and the altered IL-12 response suggests that Rip2 may be involved in IL-12 signalling either directly or indirectly. IL-12 is one of the principal cytokines regulating T-cell differentiation. We therefore analysed T-cell differentiation of *Rip2*$^{-/-}$ T cells. $CD4^+$ T cells were cultured for 4 days under either T_H1 or T_H2 conditions, and re-stimulated with plate-bound anti-CD3 antibodies for 24 h. IFN-γ production by T_H1 cells of *Rip2*$^{-/-}$ mice was reduced, although IL-4 production of *Rip2*$^{-/-}$ T_H2 cells was not affected. These results suggest that Rip2 has an important role in the differentiation of T_H1 cells but not T_H2 cells.

Altered TCR signalling has a profound influence on T_H1/T_H2 differentiation[24]. We therefore examined the response of *Rip2*$^{-/-}$ T cells on TCR stimulation with anti-CD3 antibodies. $CD4^+$ T cells of *Rip2*$^{-/-}$ mice showed severely reduced proliferation upon anti-CD3 stimulation in a dose- and time-dependent manner (Fig. 6a). IL-2 production was reduced in *Rip2*$^{-/-}$ $CD4^+$ T cells, and this defect could not be rescued by co-stimulation with anti-CD28 (Fig. 6b). As Rip2 is involved in NF-κB activation[1–4], and that NF-κB activation is required for T-cell proliferation upon TCR stimulation[25,26], we analysed NF-κB activation in *Rip2*$^{-/-}$ T cells upon anti-CD3 stimulation. Phosphorylation and degradation of IκBα was assessed by western blotting, and was reduced in *Rip2*$^{-/-}$ T cells (Fig. 6c). In addition, NF-κB activation assessed by gel-shift assay was also substantially reduced (Fig. 6d). Taken together, these data indicated that Rip2 is required for optimal activation of NF-κB and T-cell proliferation upon TCR stimulation. As inhibition of NF-κB activation can cause T_H1 deficiency *in vivo*[27], the T_H1 deficiency in *Rip2*$^{-/-}$ T cells may be attributable, at least in part, to altered TCR signalling and NF-κB activation.

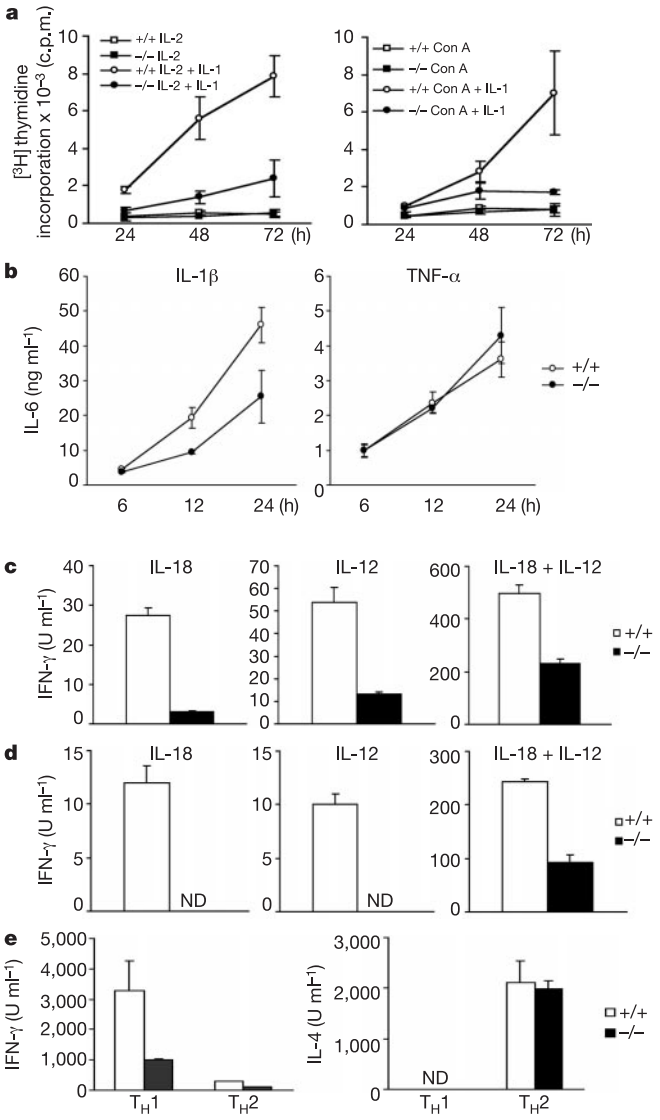

Figure 5 Rip2 is required for optimal IL-1/IL-18 receptor signalling in NK cells and T_H1 effector CD4 T cells. **a**, Proliferation of *Rip2*$^{-/-}$ thymocytes stimulated with IL-2 (2 ng ml^{-1}) or Con A (0.625 μg ml^{-1}) alone or together with IL-1β (10 ng ml^{-1}), and cultured for indicated periods. Cells were pulsed with [^{3}H]thymidine 8 h before collection. **b**, IL-6 production by *Rip2*$^{-/-}$ embryonic fibroblasts stimulated with IL-1β (left, 10 ng ml^{-1}) or TNF-α (right, 10 ng ml^{-1}) for indicated periods. **c**, Production of IFN-γ by NK cells on stimulation with IL-18 and IL-12. Splenocytes from wild-type or *Rip2*$^{-/-}$ mice were stimulated with IL-18 (10 ng ml^{-1}) or IL-12 (10 ng ml^{-1}) alone for 48 h, or with both IL-18 (10 ng ml^{-1}) and IL-12 (1 ng ml^{-1}) for 24 h. **d**, Production of IFN-γ by T_H1 cells stimulated with either IL-18 (10 ng ml^{-1}) or IL-12 (10 ng ml^{-1}) alone, or with both IL-18 and IL-12 for 24 h. **e**, T_H1/T_H2 differentiation of $CD4^+$ T cells *in vitro*. $CD4^+$ T cells from wild-type or *Rip2*$^{-/-}$ mice were stimulated with 10 μg ml^{-1} of plate-bound anti-CD3, and cultured for 4 days under T_H1 conditions (3.5 ng ml^{-1} IL-12 and 2 μg ml^{-1} anti-IL-4 antibody) or T_H2 conditions (1,000 U ml^{-1} IL-4 and 1 μg ml^{-1} anti-IFN-γ antibody). After washing, cells were counted and re-stimulated with 10 μg ml^{-1} anti-CD3 and cultured for 24 h. All experiments were performed in triplicates and were repeated three times, with equivalent results.

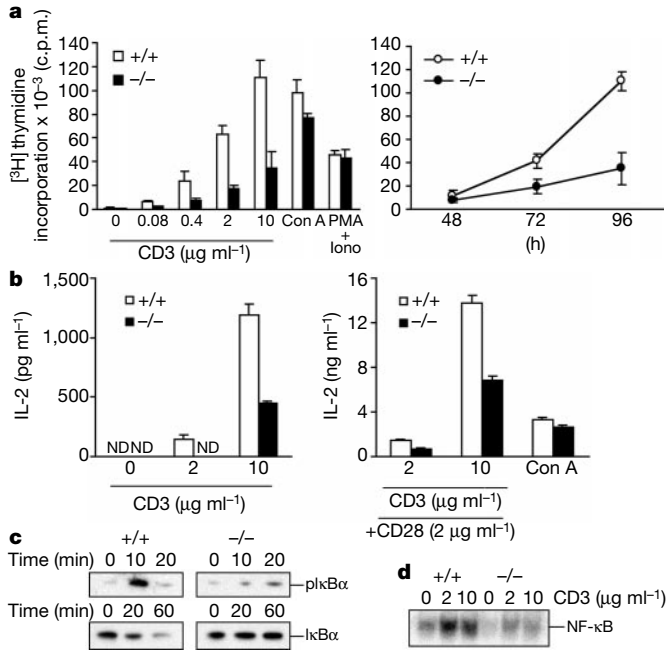

Figure 6 Rip2 is required for optimal NF-κB activation, IL-2 production and T-cell proliferation on TCR stimulation. **a**, Proliferation of *Rip2*$^{-/-}$ $CD4^+$ T cells stimulated with Concanavalin A (Con A, 2.5 μg ml^{-1}), PMA (40 ng ml^{-1}) plus ionomycin (0.5 μM) or anti-CD3 at the indicated dose (96 h), or with 10 μg ml^{-1} anti-CD3 for indicated periods in the presence of irradiated T-cell-depleted splenocytes. **b**, IL-2 production by $CD4^+$ T cells stimulated with plate-coated anti-CD3 either in the absence or presence of anti-CD28 (2 μg ml^{-1}), or with Con A (2.5 μg ml^{-1}). **c**, Phosphorylation and degradation of IκBα in splenic T cells stimulated with anti-CD3 (10 μg ml^{-1}), examined by western blotting. **d**, NF-κB activation of splenic T cells stimulated with anti-CD3 with indicated dose for 8 h, analysed by gel mobility shift assay. Experiments in **a** and **b** were performed in triplicates and were repeated three times with equivalent results.

Our results indicate that Rip2 is required for signalling through both TLRs and Nod protein family members, which are central components of the innate immune system. We also show that Rip2 is required for appropriate TCR signalling. Therefore, Rip2 is a unique kinase capable of integrating signals emanating from both the innate as well as the adaptive immune systems. In fact, a notable feature of Rip2 deficiency is that it reveals defects in signalling from various immune receptors. There are three conceivable mechanistic explanations for this. The first is that Rip2 is a direct downstream signalling molecule of each receptor. Second, it is possible that Rip2 always receives signals from Nod proteins, and that perhaps different Nod proteins are the common element downstream of these different innate and adaptive immune receptor systems, and upstream of Rip2. The third possibility is that some receptors are dependent on Rip2 via Nod proteins for signalling, and others are not. Studies involving Nod-deficient mice should help to resolve this mechanistic issue. □

Methods

Generation of Rip2-deficient mice

A murine partial complementary DNA that codes for Rip2 was obtained by polymerase chain reaction, using mouse heart first-strand cDNA (Clontech) as the template and a specific primer based on GenBank accession number AA655189 and a primer based on the human *RIP2* sequence. A 129SV/J genomic library (Stratagene) was screened with the murine *Rip2* cDNA, and two phage carrying overlapping genomic clones encompassing *Rip2* were isolated. A targeting vector was designed to replace a 4.0-kilobase genomic fragment containing the second and third exons coding for the active site aspartate residue with the *loxP*-flanked *neo*^{r} gene expression cassette. The targeting vector was linearized with *Not*I and electroporated into W9.5 embryonic stem cells. Clones resistant to G418 and gancyclovir were selected, and homologous recombination (6 out of 135 clones) was confirmed by Southern blotting. Three clones homologous for the targeted mutation were injected into C57BL/6 blastocysts, which were subsequently transferred into pseudopregnant foster mother mice.

Plasmids and reagents

The expression vectors pcDNA3-IKKβ, pcDNA3-Myc-Rip2, pcDNA3-Nod1-Flag, pcDNA3-Nod2-Flag, pEF1-BOS-β-gal and pBVI-Luc were described previously[16]. Lipopolysacchride (LPS) from *Salmonella abortus equi* and lipoteichoic acid (LTA) from *Staphylococcus aureus* were purchased from Sigma. Peptidoglycan (PGN) from *S. aureus* was from Fluka. Poly(IC) was from Amersham Pharmacia Biotech. Phosphorothioate-modified CpG oligonucleotide DNA (TCCATGACGTTCCTGACGTT) was synthesized in the Howard Hughes Medical Institute Biopolymer and W. M. Keck Biotechnology Resource Laboratory in Yale University. Human IL-1β and mouse TNF-α were from R&D mouse IL-18 was from MBL; mouse IL-12 was from Genetics Institute; mouse IL-4 was from PharMingen; and anti-CD3, anti-IL-4 or anti-IFN-γ were purified from supernatants of 2C11, 11B11 or XMG hybridoma, respectively.

Culture of bone-marrow-derived macrophages

Macrophages derived from bone marrow were prepared as described[28]. Cells were collected with cold DPBS, washed, re-suspended in DMEM, supplemented with 10% fetal calf serum, and used at a density of 2×10^5 ml^{-1} in the experiments. Cells were left untreated for at least 4 h at 37 °C in 10% CO_2 before further handling.

Listeria infection of macrophages

The cells were cultured without antibiotics, and *L. monocytogenes* (American Type Culture Collection strain 43251) was added at a multiplicity of infection (MOI) of 50. After incubation for 30 min, extracellular bacteria were removed by washing three times with DPBS. To prevent reinfection, we cultured the cells in medium containing gentamicin sulphate (50 μg ml^{-1}, Gibco BRL)

Measurement of cytokine production

Macrophages derived from bone marrow were cultured with the indicated concentration of LPS, LTA, PGN or CpG DNA for 6 h. Embryonic fibroblasts were cultured with poly(IC), LPS, IL-1β or TNF-α at the indicated concentration for the indicated periods. The concentration of IL-6, TNF-α and IP10 in culture supernatants was measured by ELISA.

Proliferation assays

We stimulated thymocytes with IL-2 (2 ng ml^{-1}) or Con A (0.625 μg/ml), in the presence or absence of IL-1β (10 ng ml^{-1}). Purified CD4$^+$ T cells were stimulated with anti-CD3 (2C11) in the presence of T-cell-depleted irradiated splenocytes. Cells were pulsed with [^{3}H]thymidine for 8 h, and its incorporation was measured with a β-plate counter (Wallac).

Cytokine production by NK cells and T cells

T_H1/T_H2 differentiated cells were washed and re-stimulated with 10 μg ml^{-1} anti-CD3, and cultured for 24 h. IFN-γ and IL-4 in the supernatant was measured. Total splenocytes and T_H1 cells were stimulated with IL-18 (10 ng ml^{-1}), IL-12 (10 ng ml^{-1}) or a combination of both. We measured the concentration of IFN-γ and IL-4 by ELISA. For IL-2 production, purified CD4$^+$ T cells were stimulated with plate-bound anti-CD3 in the presence or absence of anti-CD28 (2 μg ml^{-1}) at the indicated concentration for 24 h, and the level of IL-2 in the supernatant was measured by ELISA.

NF-κB activation assay

NF-κB activation assays were carried out as described[1].

Western blot analysis and immunoprecipitation

Cell lysis and blotting were carried out as described[29]. Membranes were blotted with antibodies to Rip2 (Cayman), IκB, JNK, p38, ERK1/2 (phosphorylated and unphosphorylated forms for each; Cell signaling), IRAK-1, TRAF6 (both Santa Cruz), and MyD88 (StressGen). We carried out immunoprecipitation as described[29] using an anti-Flag monoclonal antibody (Sigma).

Northern blot analysis

Macrophages derived from bone marrow were stimulated with 10 ng ml^{-1} LPS for the indicated periods. Preparation of total RNA samples and northern blot analysis were performed as described previously[29].

T-cell stimulation for NF-κB activation

Purified splenic T cells were incubated with anti-CD3 antibodies at the indicated concentration for 30 min on ice. After washing, cells were incubated with anti-hamster immunoglobulin-γ antibody (100 μg ml^{-1}; Vector) at 37 °C for the indicated periods. Cytoplasmic and nuclear extracts were used for western blot analysis and gel mobility shift assay using a [^{32}P]dCTP-labelled probe specific for NF-κB (5′-GGAGTTGAGGGGACT TTCCCAGGC-3′).

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Competing interests statement

The authors declare that they have no competing financial interests.

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An amino-acid taste receptor

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The sense of taste provides animals with valuable information about the nature and quality of food. Mammals can recognize and respond to a diverse repertoire of chemical entities, including sugars, salts, acids and a wide range of toxic substances[1]. Several amino acids taste sweet or delicious (umami) to humans, and are attractive to rodents and other animals[2]. This is noteworthy because L-amino acids function as the building blocks of proteins, as biosynthetic precursors of many biologically relevant small molecules, and as metabolic fuel. Thus, having a taste pathway dedicated to their detection probably had significant evolutionary implications. Here we identify and characterize a mammalian amino-acid taste receptor. This receptor, T1R1+3, is a heteromer of the taste-specific T1R1 and T1R3 G-protein-coupled receptors. We demonstrate that T1R1 and T1R3 combine to function as a broadly tuned L-amino-acid sensor responding to most of the 20 standard amino acids, but not to their D-enantiomers or other compounds. We also show that sequence differences in T1R receptors within and between species (human and mouse) can significantly influence the selectivity and specificity of taste responses.

T1Rs and T2Rs are two families of G-protein-coupled receptors (GPCRs) selectively expressed in subsets of taste receptor cells[3–11]. T2Rs are involved in bitter taste detection[4,5], and T1R2 and T1R3 combine to function as a sweet taste receptor[7]. To identify taste receptors involved in amino-acid detection, we used an expression screening strategy similar to that used in the characterization of bitter and sweet taste receptors. Candidate receptors were expressed in human embryonic kidney (HEK) cells containing the $G\alpha_{16}$–$G\alpha_{Z}$ and $G\alpha_{15}$ promiscuous G proteins[12,13], and assayed for stimulus-evoked changes in intracellular calcium. In this system, receptor activation leads to activation of phospholipase Cβ (PLC-β) and release of calcium from internal stores, which can be monitored at the single-cell level using calcium-indicator dyes[5,7,14].

Because T1R taste receptors are distantly related to GPCRs that recognize the amino acids glutamate[15] (metabotropic glutamate receptors, mGluRs), GABA[16] (γ-aminobutyric acid; GABA-B receptors) and arginine[17] (the R5-24 receptor), we began by testing members of the T1R family. Patterns of T1R expression define at least three distinct cell types: cells co-expressing T1R2 and T1R3 (T1R2+3, a sweet receptor), cells co-expressing T1R1 and T1R3 (T1R1+3) and cells expressing T1R3 alone[7]. First, we assayed responses of the T1R2+3 sweet taste receptor to all 20 standard and various D-amino acids. Several D-amino acids that taste sweet to humans, and are attractive to mice, trigger robust activation of the T1R2+3 sweet taste receptor (Fig. 1a, b). However, none of the tested L-amino acids activate this receptor.

Mouse T1R1 and T1R3 were transfected alone or in combination and tested for stimulation by L-amino acids. Individual receptors showed no responses. In contrast, T1R1 and T1R3 combine to function as a broadly tuned L-amino-acid receptor, with most amino acids that are perceived as sweet (for example, alanine, glutamine, serine, threonine and glycine[2]) activating T1R1+3 (Fig. 1). The responses are strictly dependent on the combined presence of T1R1 and T1R3, and are highly selective for L-amino acids; D-amino acids and other natural and artificial sweeteners did not activate the T1R1+3 receptor combination. These results substantiate T1R1+3 as a receptor for L-amino acids, and provide a striking example of heteromeric GPCR receptors radically altering their selectivity by a combinatorial arrangement of subunits.

If T1R1+3 functions as a major L-amino acid taste sensor *in vivo*, we might expect its cell-based behaviour to recapitulate some of the physiological properties of the *in vivo* receptor. Nerve recordings in rats have shown that taste responses to L-amino acids are considerably potentiated by purine nucleotides such as inosine monophosphate (IMP)[18]. To assay the effect of IMP, HEK cells expressing the T1R1+3 receptor combination were stimulated with amino acids in the presence or absence of IMP. Indeed, T1R1+3 responses to nearly all L-amino acids were dramatically enhanced by low doses of IMP (Figs 1 and 2a); this effect increased over a range of 0.1–10 mM (Fig. 2b). However, IMP alone elicited no response, even at the highest concentration tested in our assays, and it had no effect on responses mediated by T1R2+3 (either to sweeteners or to L- and D-amino acids; data not shown).

T1R1+3 is prominently expressed in fungiform taste buds[7], which are innervated by chorda tympani fibres. Therefore, we stimulated mouse fungiform papillae at the front of the tongue with various amino acids in the presence or absence of IMP, and recorded tastant-induced spikes from the chorda tympani nerve. As expected, nerve responses to L-amino acids were significantly enhanced by IMP[18] (Fig. 3). However, IMP had no significant effect on responses to D-amino acids or to non-amino-acid stimuli.

Genetic studies of sweet tasting have identified a single principal locus in mice influencing responses to several sweet substances (the *Sac* locus[19,20]). Sac 'taster' mice are about fivefold more sensitive to sucrose, saccharin and other sweeteners than Sac non-tasters. *Sac* codes for T1R3[7–11,21]. There are two amino-acid differences that define taster and non-taster alleles[7,9,10]. One of these changes, I60T, introduces a potential glycosylation site that was proposed to eliminate receptor function by preventing receptor dimerization[10]. This poses a conundrum because responses to L-amino acids are not influenced by the *Sac* locus[7,22] (and data not shown). Thus, if T1R3 functions as the common partner of the sweet and amino-acid receptors, we reasoned that the T1R3 non-taster allele must selectively affect the T1R2+3 combination.

We examined the effect of the *Sac* non-taster allele on T1R1 and