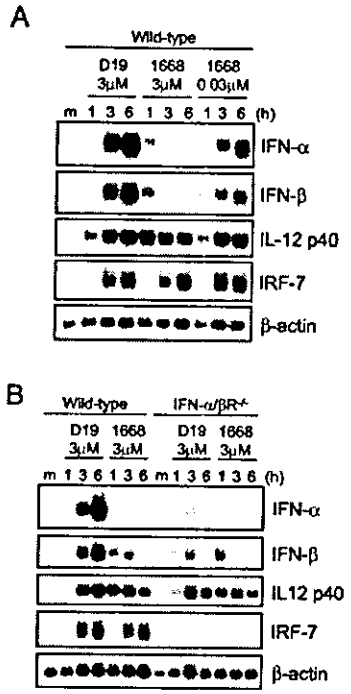

FIGURE 5. Northern blot analysis of Flt3L-BMDCs stimulated with D19 or ODN1668. Flt3L-BMDCs from wild-type or IFN-$\alpha/\beta R^{-/-}$ mice were stimulated with CpG DNAs for the indicated periods. Total RNAs were extracted and subjected to Northern blot analysis.

(18–20). We found that A/D-type CpG DNAs also stimulated $B220^+CD11c^{dull}$ cells to induce IFN-α production. $B220^+CD11c^{dull}$ cells could also produce significant, but smaller, amounts of IFN-α by conventional CpG DNAs only at low doses, while $B220^-CD11c^{high}$ cells did not (data not shown). Interestingly, although $B220^-CD11c^{high}$ cells secreted similar amounts of IL-12 in response to the two types of CpG DNAs, $B220^+CD11c^{dull}$ cells produced smaller amounts of IL-12 when stimulated with A/D-type CpG DNAs than with conventional CpG DNAs. Meanwhile, costimulatory molecule expression in $B220^+CD11c^{dull}$ and $B220^-CD11c^{high}$ cells was comparatively up-regulated by both types of CpG DNAs. These results suggest that differential responses to conventional or A/D-type CpG DNAs are observed in cytokine-producing ability, but not in the ability to enhance costimulatory molecule expression. Notably, $B220^-CD11c^{high}$ cells, which cannot produce IFN-α, responded to the two types of CpG DNAs in an indistinguishable manner. Thus, the differential ability of DCs to produce cytokines in response to conventional or A/D-type CpG DNAs is based on the characteristics of $B220^+CD11c^{dull}$ cells.

At present we do not know how TLR9 can transduce such differential activity depending on the ligand. Klinman et al. (7) have shown that TLR9 overexpression can render a human kidney cell line responsive to conventional CpG DNAs, but not to A/D-type CpG DNAs. This suggests that TLR9 is not sufficient for transducing the A/D-type CpG DNA signal, and that another molecule(s) cooperatively functions to induce IFN-α production with TLR9. It is assumed that oligodeoxynucleotides are incorporated into cells through a pathway not requiring any specific sequences and that only CpG DNAs can bind to TLR9 and trigger TLR9 signaling in the endosome (10, 28). However, confocal microscopic analysis revealed that conventional and A/D-CpG DNAs are destined for different cell compartments (29). Such differential behavior of CpG DNAs might lead to the induction of distinct cytokines. It is also possible that differential TLR9 responses are caused by coligation of other transmembrane proteins. In this context, it is noteworthy that scavenger receptor A (SR-A) can bind to the polyG stretch and that SR-A ligands can inhibit the binding of CpG DNAs containing the polyG stretch to splenic $CD11c^+$ DCs (30). However, it remains unknown whether SR-A is involved in DC responses caused by A/D-type CpG DNAs.

DNA-PKcs was suggested as a signaling molecule for CpG DNA-induced immune responses (24). However, a recent report has shown that DNA-PKcs is not essential for CpG DNA responses (31). Our present results are consistent with the latter report and further show that the enzyme is dispensable not only for conventional, but also for A/D-type, CpG DNAs-induced signaling. In addition, $MyD88^{-/-}$ and $TLR9^{-/-}$ cells lacked any response to either type of CpG DNAs (Fig. 3). Thus, CpG DNAs can manifest their multiple immunostimulatory functions through the TLR9-MyD88-dependent pathway.

Viral infection can induce type I IFN production that is vigorously amplified by type I IFN itself (32, 33). IFN-$\alpha/\beta R^{-/-}$ cells decreased their ability to produce IFN-α in response to A/D-type CpG DNAs, indicating that type I IFN signaling is also involved in TLR9-induced IFN-α production. IRF7 mRNA can be induced by type I IFN. The induction was suggested to be critical for IFN-α production through the analysis of IRF-9-deficient mice that lack IRF7 mRNA induction (27). However, wild-type DCs could produce similarly increased levels of IRF7 mRNA expression in response to 3 μM ODN1668, although IFN-α production was profoundly decreased (Fig. 5). Thus, IRF7 mRNA induction is not sufficient for TLR9-induced IFN-α production.

In response to 3 μM ODN1668, low levels of type I IFN mRNA expression can be induced at 1 h, but the induction rapidly declines at later time points. Meanwhile, at 0.03 μM ODN1668 the induction comes later and reaches comparable levels as with D19. These results suggest the possibility that a high concentration of ODN1668 induces unknown negative feedback mechanism resulting in the abolishment of type I IFN gene expression. Further studies are necessary to clarify how TLR9 induces differential responses depending on its ligands.

Among TLR family members, TLR7 is closely related to TLR9 based on their molecular structures. In humans, TLR7 and TLR9 are expressed on a subset of DCs, PDC. Activation of TLR7 or TLR9 enhances the survival of DCs and the expression of surface molecules, such as costimulatory molecules and MHC class II. Thus, TLR7 and TLR9 have common features in their molecular structures and functions. While TLR9 is expressed exclusively on PDC, TLR7 is also expressed on another subset, myeloid DC. Experiments with TLR7 ligands clarified that TLR7 signaling can differentially induce IFN-α and IL-12 from PDC and myeloid DC, respectively (34). The mechanism is unknown at present, but it is intriguing that murine TLR9 and the human TLR7 systems are well conserved in differential cytokine inducibility depending on DC subsets. Another intriguing point is that murine $B220^+CD11c^{dull}$ cells do not produce high levels of IL-12 in response to D19, although they can in response to ODN1668. This is in contrast to the human TLR7 system, because human PDC cannot produce IL-12 in response to any stimuli. IFN-α can inhibit IL-12 production (35), but that cannot account for the inability of A/D-type CpG DNA-stimulated $B220^+CD11c^{dull}$ cells to produce IL-12, because costimulation with IFN-α and ODN1668 did not decrease IL-12 production from $B220^-CD11c^{high}$ cells (data not shown). Furthermore, type I IFN-neutralizing Abs could not increase IL-12 production from D19-treated $B220^+CD11c^{dull}$ cells (data not shown). Thus, it is unlikely that IFN-α production induced by D19 prevents $B220^+CD11c^{dull}$ cells from producing IL-12.

The present study has revealed subset-dependent responses of murine DCs to distinct types of CpG DNA. Further clarification of the underlying molecular mechanisms should contribute to elucidating how DCs are activated by CpG DNAs and to developing efficient vaccination strategies with CpG DNAs.

Acknowledgments

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References

1. Wagner, H. 1999. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol. 73:329.*
2. Yamamoto, S., T. Yamamoto, and T. Tokunaga. 2000. The discovery of immunostimulatory DNA sequence. *Springer Semin. Immunopathol. 22:11.*
3. Kreig, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol. 20:709.*
4. Verthelyi., D., K. J. Ishii, M. Gursel, F. Takeshita, and D. M. Klinman. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. *J. Immunol. 166:2372.*
5. Krug, A., S. Rothenfusser, V. Hornung, B. Jahrsdorfer, S. Blackwell, Z. K. Ballas, S. Endres, A. M. Krieg, and G. Hartmann. 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells. *Eur. J. Immunol. 31:2154.*
6. Ballas, Z. K., A. M. Krieg, T. Warren, and G. L. Weiner. 2001. Divergent therapeutic and immunological effects of oligonucleotides with distinct CpG motifs. *J. Immunol. 167:4878.*
7. Klinman, D. M., F. Takeshita, I. Gursel, C. Leifer, K. J. Ishii, D. Verthelyi, and M. Gursel. 2002. CpG DNA: recognition by and activation of monocytes. *Microbes Infect. 4:897.*
8. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, et al. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*
9. Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA 98:9237.*
10. Takeshita, F., C. A. Leifer, I. Gursel, K. J. Ishii, S. Takeshita, M. Gursel, and D. M. Klinman. 2001. Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J. Immunol. 167:3555.*
11. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity. 9:143.*
12. Gao, Y., J. Chaudhuri, C. Zhu, L. Davidson, D. T. Weaver, and F. W. Alt. 1998. A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for KU in V(D)J recombination. *Immunity 9:367.*
13. Krieg, A. M., A. K. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature. 374:546.*
14. Gilliet, M., A. Boonstra, C. Paturel, S. Antonenko, X. L. Xu, G. Trinchieri, A. O'Garra, and Y. J. Liu. 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 195:953.*
15. Kawai T, O. Takeuchi, T. Fujita, J. Inoue, P. F. Muhlradt, S. Sato, K. Hoshino, and S. Akira. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol. 167:5887.*
16. Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, et al. 2000. Distinct and essential roles of transcription factors IRF-3 and IRF7 in response to viruses for IFN-α/β gene induction. *Immunity. 13:539.*
17. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol. 2:151.*
18. Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Brière, et al. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol. 2:1144.*
19. Nakano, H., M. Yanagita, and M. D. Gunn. 2001. CD11c$^+$B220$^+$Gr-1$^+$ cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J. Exp. Med. 194:1171.*
20. Martin, P., G. M. Del Hoyo, F. Anjuère, C. F. Arias, H. H. Vargasm, A. Fernández-L, V. Parrillas, and C. Ardavin. 2002. Characterization of a new subpopulation of mouse CD8α^+ B220$^+$ dendritic cells endowed with type 1 interferon production capacity and tolerogenic potential. *Blood 100:383.*
21. Nikolic, T., G. M. Dingjan, P. J. Leenen, and R. W. Hendriks. 2002. A subfraction of B220$^+$ cells in murine bone marrow and spleen does not belong to the B cell lineage but has dendritic cell characteristics. *Eur. J. Immunol. 32:686.*
22. Häcker, H., R. M. Vabulas, O. Takeuchi, K. Hoshino, S. Akira, and H. Wagner. 2000. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J. Exp. Med. 192:595.*
23. Kaisho, T., and S. Akira. 2002. Toll-like receptors as adjuvant receptors. *Biochim. Biophys. Acta 1589:13.*
24. Chu, W., X. Gong, Z. Li, K. Takabayashi, H. Ouyang, Y. Chen, A. Lois, D. J. Chen, G. C. Li, M. Karin, et al. 2000. DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell 103:909.*
25. Kurimasa A, H. Ouyang, L. J. Dong, S. Wang, X. Li, C. Cordon-Cardo, D. J. Chen, and G. C. Li. 1999. Catalytic subunit of DNA-dependent protein kinase: impact on lymphocyte development and tumorigenesis. *Proc. Natl. Acad. Sci. USA 96:1403.*
26. Marié, I., J. E. Durbin, and D. E. Levy. 1998. Differential viral induction of distinct interferon-α genes by positive feedback through interferon regulatory factor-7. *EMBO J. 17:6660.*
27. Sato, M., N. Hata, M. Asagiri, T. Nakaya, T. Taniguchi, and N. Tanaka. 1998. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF7. *FEBS Lett. 441:106.*
28. Ahmad-Nejad, P., H. Häcker, M. Rutz, S. Bauer, R. M. Vabulas, and H. Wagner. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur. J. Immunol. 32:1958.*
29. Gürsel, M., D. Verthelyi, I. Gürsel, K. J. Ishii, and D. M. Klinman. 2002. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *J. Leukocyte Biol. 71:813.*
30. Lee, S. W., M. K. Song, K. H. Baek, Y. Park, J. K. Kim, C. H. Lee, H. K. Cheong, C. Cheong, and Y. C. Sung. 2000. Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. *J. Immunol. 165:3631.*
31. Ishii, K. J., F. Takeshita, I. Gursel, M. Gursel, J. Conover, A. Nussenzweig, and D. M. Klinman. 2002. Potential role of phosphatidylinositol 3 kinase, rather than DNA-dependent protein kinase, in CpG DNA-induced immune activation. *J. Exp. Med. 196:269.*
32. Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol. 19:623.*
33. Levy, D. E., I. Marié, E. Smith, and A. Prakash. 2002. Enhancement and diversification of IFN induction by IRF7-mediated positive feedback. *J. Interferon Cytokine Res. 22:87.*
34. Ito, T., R. Amakawa, T. Kaisho, H. Hemmi, K. Tajima, K. Uehira, Y. Ozaki, H. Tomizawa, S. Akira, and S. Fukuhara. 2002. Interferon-α and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. *J. Exp. Med. 195:1507.*
35. Biron, C. A. 2001. Interferons α and β as immune regulators: a new look. *Immunity 14:661.*

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TRAM is specifically involved in the Toll-like receptor 4–mediated MyD88-independent signaling pathway

Masahiro Yamamoto[1], Shintaro Sato[2], Hiroaki Hemmi[1], Satoshi Uematsu[1], Katsuaki Hoshino[3], Tsuneyasu Kaisho[1,3], Osamu Takeuchi[1], Kiyoshi Takeda[1,2] & Shizuo Akira[1,2]

Recognition of pathogens by Toll-like receptors (TLRs) triggers innate immune responses through signaling pathways mediated by Toll–interleukin 1 receptor (TIR) domain–containing adaptors such as MyD88, TIRAP and TRIF. MyD88 is a common adaptor that is essential for proinflammatory cytokine production, whereas TRIF mediates the MyD88-independent pathway from TLR3 and TLR4. Here we have identified a fourth TIR domain–containing adaptor, TRIF-related adaptor molecule (TRAM), and analyzed its physiological function by gene targeting. TRAM-deficient mice showed defects in cytokine production in response to the TLR4 ligand, but not to other TLR ligands. TLR4- but not TLR3-mediated MyD88-independent interferon-β production and activation of signaling cascades were abolished in TRAM-deficient cells. Thus, TRAM provides specificity for the MyD88-independent component of TLR4 signaling.

TLRs discriminate among molecular patterns associated with microbial components. TLRs are abundantly expressed on professional antigen-presenting cells such as macrophages and dendritic cells, and serve as an important link between the innate and adaptive immune responses[1,2]. So far, genes encoding ten TLRs (TLR1–TLR10) have been found in the human genome, and each TLR recognizes specific pathogen-associated patterns. TLR1 recognizes triacylated lipoprotein[3]. TLR2 is essential for recognition of peptidoglycan (PGN), the main Gram-positive bacterial cell wall component[4]. TLR3 is involved in recognition of double-stranded RNA, which is generated in the life cycle of RNA viruses[5]. TLR4 is a receptor for lipopolysaccharide (LPS), a Gram-negative cell wall component[6,7]. TLR5 recognizes flagellin, a component of bacterial flagella[8]. TLR6 is required for recognition of diacylated lipoprotein[9], whereas TLR7 is crucial for recognition of imidazoquinoline, an antiviral synthetic compound, and its derivative, R-848 (ref. 10). TLR9 is a receptor for bacterial unmethylated CpG DNA[11].

Intracellular signaling pathways of TLRs are elicited from the TIR domain, which is conserved among the cytoplasmic regions of TLRs. A cytoplasmic molecule, MyD88, contains a TIR domain and a death domain. The death domain of MyD88 is required for interaction with other death domain–containing molecules such as IRAK-1 and IRAK-4 (refs. 12–14). The TIR domain is reportedly required for the formation of dimers with other TIR domain–containing receptors or adaptors. Indeed, MyD88-deficient mice show neither splenocyte proliferation nor production of proinflammatory cytokines in response to all TLR ligands and interleukin 1 (IL-1), indicating that MyD88 is essential for the immune responses through all TLRs and the IL-1 receptor (IL-1R)[15]. However, a TLR3 ligand, poly(I:C), and the TLR4 ligand, LPS, still stimulate the expression of certain genes, such as the gene encoding interferon-β (IFN-β), in MyD88-deficient mice. Induction of IFN-β expression leads to maturation of dendritic cells and subsequent expression of IFN-inducible genes[16–18]. These observations indicated that TLR signaling is composed of at least two pathways: a MyD88-dependent pathway that leads to the production of proinflammatory cytokines, and a MyD88-independent pathway associated with the induction of IFN-inducible genes and maturation of dendritic cells. Moreover, the specificity of the MyD88-dependent signaling pathways through all TLRs is provided by TIRAP, the second TIR domain–containing adaptor discovered[19,20]. TIRAP-deficient mice show defects in activation of the MyD88-dependent signaling pathway through TLR2 and TLR4, but not other TLRs[21,22].

Although the precise molecular mechanisms of the MyD88-independent signaling pathway are unknown, identification of another TIR domain–containing molecule, TRIF[23,24] (also called TICAM-1), and genetic evidence from mice carrying a mutation in this gene demonstrated that TRIF is crucial in the MyD88-independent signaling pathway shared by TLR3 and TLR4 (refs. 25,26). Furthermore, two noncanonical IκB kinases (IKKs), IKK-*i* (also called IKKε) and TBK1 (also called T2K), interact with TRIF, activate IRF-3 and, finally, lead to IFN-β induction[27,28].

Two more TIR domain–containing adaptors have been identified in the human genome. The physiological function of the first, SARM (for sterile alpha motif (SAM) and Armadillo motif (ARM) domain–containing protein), in TLR–IL-1R signaling remains unclear[29,30].

[1]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, and [2]ERATO, Japan Science and Technology Corporation, 3-1 Yamada-oka, Suita Osaka 565-0871, Japan. [3]RIKEN Research Center for Allergy and Immunology, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. Correspondence should be addressed to S.A. (sakira@biken.osaka-u.ac.jp).

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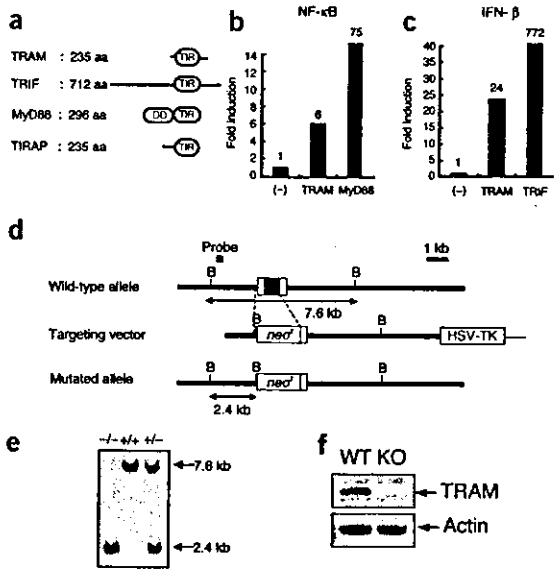

Figure 1 Cloning and characterization of human TRAM and targeted disruption of the gene encoding mouse TRAM. (**a**) Comparison of the structures of human TRAM, TRIF, MyD88 and TIRAP. Domains were determined with the BLAST program. Lengths are indicated in amino acids (aa). DD, death domain; TIR, TIR domain. (**b**,**c**) 293 cells were transiently transfected with 1 μg TRAM, MyD88, TRIF or empty vector (–), plus 0.1 μg NF-κB reporter (**b**) or the IFN-β promoter luciferase reporter (**c**). Then, 24 h after transfection, luciferase activity was measured. Numbers over each column indicate the 'fold induction', which for empty vector was normalized to onefold. (**d**) The structures of the gene encoding TRAM (top), the targeting vector (middle) and the predicted disrupted gene (bottom). Gray box, coding exon. B, *Bam*HI. (**e**) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tail tissue, digested with *Bam*HI, separated by electrophoresis and hybridized with the radiolabeled probe shown in **d**. Southern blot analysis detected a single 7.6-kb band for wild-type mice (+/+), a 2.4-kb band for homozygous TRAM-deficient mice (–/–) and both bands for heterozygous mice (+/–). (**f**) Cell lysates prepared from macrophages were immunoprecipitated and immunoblotted with anti-TRAM. Bottom, the same lysates blotted with anti-actin to monitor protein expression. WT, wild-type; KO, TRAM-deficient.

Previous *in vitro* analysis of the other, TRAM (also called TIRP)[31], indicates that its ectopic expression activates NF-κB, as does MyD88, TIRAP and TRIF. However, it does not activate the promoter for the gene encoding IFN-β, unlike TRIF. Dominant negative mutants of this protein inhibit the NF-κB activation through IL-1R, but not through TLRs, indicating that TRAM is a specific adaptor protein in the IL-1R-mediated MyD88-dependent signaling pathway. However, the function of TRAM *in vivo* remains to be clarified.

Here we report the generation of TRAM-deficient mice and detailed analysis of TLR–IL-1R signaling in these mutant mice. TRAM-deficient mice showed normal responses to ligands for TLR2, TLR7, TLR9 and IL-1β, but defective macrophage responses to the TLR4 ligand in cytokine production and B cell activation. Furthermore, activation of the TLR4-mediated MyD88-independent, but not MyD88-dependent, signaling cascade was abolished in TRAM-deficient mice. Although this phenotype was reminiscent of that of TRIF-deficient mice, which lack activation of the MyD88-independent pathway in both TLR3 and TLR4 signaling, TRAM-deficient mice showed a normal response to the TLR3 ligand. These results indicate that TRAM is an adaptor molecule that provides specificity for the MyD88-independent pathway of TLR4 signaling.

RESULTS

Identification of TRAM

The TIR domain–containing adaptor TRIF was identified by a database search analysis[23] (Fig. 1a). During this analysis, we identified another TIR domain–containing molecule. The TIR domain of this molecule showed greater homology with the TIR domain of TRIF than with that of MyD88 or TIRAP (data not shown). The gene encoding has an open reading frame of 708 base pairs (bp) that encodes 235 amino acids. Ectopic expression of TRAM in 293 cells activated luciferase reporters containing the NF-κB–dependent promoter or the promoter for the gene encoding IFN-β, albeit at low amounts compared with that of MyD88- and TRIF-mediated activation, respectively (Fig. 1b,c). Thus, *in vitro* studies indicated that TRAM is involved in TLR–IL-1R signaling pathways.

Generation of TRAM-deficient mice

To elucidate the physiological function of TRAM, we generated TRAM-deficient mice by gene targeting. The gene encoding mouse TRAM consists of one exon. We constructed the targeting vector to replace the entire exon with a neomycin-resistance gene cassette (Fig. 1d). We

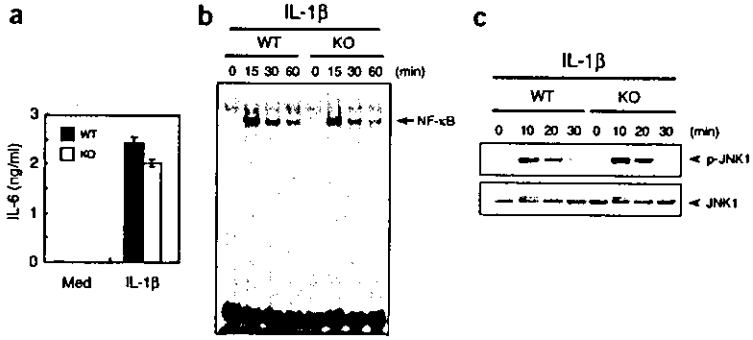

Figure 2 IL-1-induced responses in TRAM-deficient cells. (**a**) Embryonic fibroblasts from wild-type and TRAM-deficient mice were stimulated with 100 ng/ml of IL-1β. Supernatants were collected 24 h later for IL-6 analysis by ELISA. Data represent means ± s.d. of triplicate samples. N.D., not detected. (**b**) Embryonic fibroblasts were stimulated with 100 ng/ml of IL-1β (time, above lanes). Nuclear extracts were prepared, and NF-κB DNA-binding activity was determined by electrophoretic mobility shift assay with an NF-κB-specific probe. (**c**) JNK activation was also determined by immunoblot of cell extracts with anti-phospho-JNK (p-JNK). WT, wild-type; KO, TRAM-deficient.

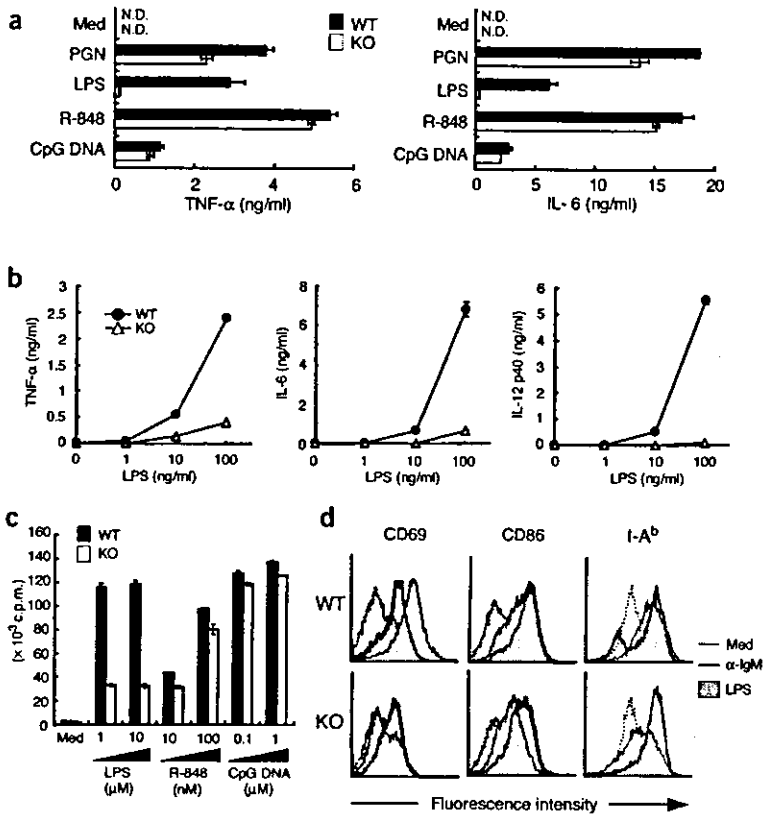

Figure 3 Impaired TLR4-mediated cytokine production and B cell activation in TRAM-deficient mice. (a) Peritoneal macrophages from TRIF-deficient mice or wild-type mice were left unstimulated (Med) or were stimulated with 10 μg/ml of PGN, 100 ng/ml of LPS, 100 nM R-848 or 1 μM CpG DNA in the presence of 30 ng/ml of IFN-γ. Supernatants were collected 24 h later for TNF (left) and IL-6 (right) analysis by ELISA. Data represent means ± s.d. of triplicate samples. N.D., not detected. (b) Peritoneal macrophages were stimulated for 24 h with LPS (concentrations, horizontal axes) in the presence of 30 ng/ml of IFN-γ, and TNF (left), IL-6 (middle) and IL-12 p40 (right) in supernatants was measured by ELISA. Data represent means ± s.d. of triplicate samples. (c) Proliferation of splenocytes stimulated with LPS, R-848 or CpG DNA. Splenocytes were cultured for 24 h with LPS, R-848 or CpG DNA (concentrations, horizontal axes; wedges indicate increasing concentrations). Samples were pulsed with [^{3}H]thymidine (1 μCi) for the last 12 h, then [^{3}H]thymidine incorporation was measured with a scintillation counter. (d) Splenic B220-positive cells were cultured with 10 μg/ml of LPS or 10 μg/ml of anti-IgM (α-IgM). After 36 h of culture, cells were collected and stained with biotin-conjugated anti-CD69, anti-CD86 or anti-I-A^b followed by phycoerythrin-conjugated streptavidin. WT, wild-type; KO, TRAM-deficient; Med, medium.

microinjected two correctly targeted embryonic stem cell clones into C57BL/6 blastocysts to generate chimeric mice. We crossed male chimeric mice with female C57BL/6 mice, and monitored transmission of the mutated allele by Southern blot analysis (Fig. 1e). We then interbred heterozygous mice to produce offspring carrying a null mutation of the gene encoding TRAM. TRAM-deficient mice were born at the expected mendelian ratio and showed no developmental abnormalities. To confirm the disruption of the gene encoding TRAM, we analyzed total RNA from wild-type and TRAM-deficient lung fibroblast cells by RNA blot. We found no transcripts for TRAM in RNA from TRAM-deficient mice (data not shown). Immunoprecipitation analysis of macrophages further confirmed that disruption of the gene encoding TRAM abolished expression of TRAM protein (Fig. 1f).

TRAM is not involved in IL-1R signaling

As a previous *in vitro* study showed that TRAM (also known as TIRP) is involved in the IL-1R-mediated MyD88-dependent pathway[31], we first tested whether IL-1R signaling was impaired in TRAM-deficient cells. We measured production of IL-6 by mouse embryonic fibroblasts from wild-type and TRAM-deficient mice in response to IL-1β by enzyme-linked immunosorbent assay (ELISA; Fig. 2a). IL-1β-induced IL-6 production was normal in TRAM-deficient mouse embryonic fibroblasts. We next analyzed activation of NF-κB and the kinase JNK1 in response to IL-1β (Fig. 2b,c). IL-1β stimulation activated NF-κB and JNK1 activation equivalently in both wild-type and TRAM-deficient mouse embryonic fibroblasts, demonstrating that TRAM is not involved in the IL-1R-mediated signaling pathway.

TLR4-mediated cytokine production and B cell activation

We next analyzed the production of proinflammatory cytokines such as tumor necrosis factor (TNF) and IL-6 by peritoneal macrophages in response to various TLR ligands, including PGN, LPS, R-848 and CpG DNA. Wild-type and TRAM-deficient macrophages produced similar amounts of IL-6 and TNF when stimulated with PGN, R-848 or CpG DNA. However, LPS-induced production was reduced in TRAM-deficient cells (Fig. 3a). Wild-type cells produced TNF, IL-6 and p40 subunit of IL-12 in response to LPS stimulation in a dose-dependent way (Fig. 3b). In contrast, LPS-induced production in TRAM-deficient cells was impaired. Thus, TRAM-deficient mice showed defective responses in terms of LPS-induced cytokine production. Next, we analyzed splenocyte proliferation in response to stimulation with LPS, R-848 or CpG DNA. Proliferation of wild-type and TRAM-deficient splenocytes in response to R-848 and CpG DNA was similar. However, TRAM-deficient splenocytes showed defective proliferation after LPS stimulation (Fig. 3c). We next analyzed surface expression of CD69, CD86 and major histocompatibility complex class II molecules on B220-positive splenocytes by flow cytometry after stimulation with LPS or antibody to IgM (anti-IgM). Both wild-type and TRAM-deficient B220-positive cells showed similar CD69, CD86 and major histocompatibility complex class II up-regulation in response to anti-IgM (Fig. 3d). However, LPS-induced up-regulation of these molecules was perturbed in TRAM-deficient cells. Therefore, these data indicated that TLR4-mediated, but not other TLR-mediated, responses were impaired in TRAM-deficient mice.

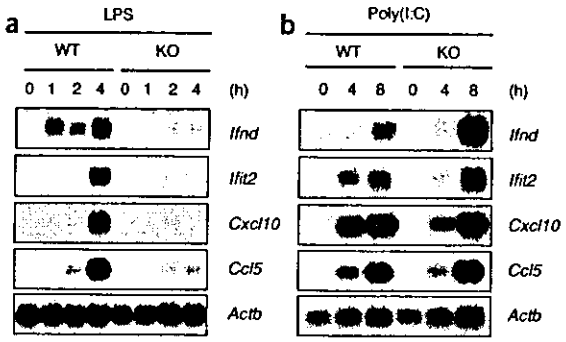

Figure 4 Defects in TLR4-mediated MyD88-independent responses in TRAM-deficient mice. Peritoneal macrophages were stimulated with 100 ng/ml of LPS (a) or 50 μg/ml of poly(I:C) (b). Total RNA (10 μg) was extracted and analyzed by RNA blot for expression of *Ifnb* (IFN-β), *Ifit2* (ISG54), *Cxcl10* (IP-10), *Ccl5* (RANTES) and *Actb* (β-actin). Stimulation times are above each lane. WT, wild-type; KO, TRAM-deficient.

TLR4-mediated MyD88-independent responses

TLR3 and TLR4 ligands induce IFN-β production and subsequent expression of IFN-inducible genes in a MyD88-independent way[5,17,18]. The impaired TLR4-mediated cytokine production and B cell activation in TRAM-deficient mice prompted us to investigate whether the MyD88-independent responses were also impaired in these mice. We analyzed expression of the gene encoding IFN-β and IFN-inducible genes in both wild-type and TRAM-deficient mice by RNA blot. LPS stimulated expression of the gene encoding IFN-β and IFN-inducible genes, including *Ifit2* (encoding ISG54), *Cxcl10* (encoding IP-10) and *Ccl5* (encoding RANTES), in wild-type peritoneal macrophages. However, LPS-induced expression of these genes was impaired in TRAM-deficient cells (Fig. 4a). As for TLR3 responses, both wild-type and TRAM-deficient peritoneal macrophages showed similar amounts of gene expression after poly(I:C) stimulation (Fig. 4b). These data indicated that TRAM deficiency affected the MyD88-independent responses mediated by TLR4.

TLR4-mediated MyD88-independent signal transduction

LPS induction of *Cxcl10* mRNA is dependent on signal transducer and activator of transcription 1 (STAT-1), as demonstrated with STAT-1-deficient mice, which show a reduction in *Cxcl10* mRNA expression in LPS-stimulated macrophages compared with that of wild-type cells[32–34]. In wild-type macrophages, the tyrosine residue at position 701 of STAT-1 was phosphorylated after 2 h of LPS stimulation. However, this typrosine remained unphosphorylated in TRAM-deficient macrophages (Fig. 5a). In contrast, poly(I:C) stimulation resulted in similar degrees of STAT-1 phosphorylation in both wild-type and TRAM-deficient cells (Fig. 5b). Given that STAT-1 phosphorylation is IFN-β dependent[34], we investigated the activation of signaling molecules required for IFN-β production. The transcription factor IRF-3 is essential for LPS- and poly(I:C)-mediated IFN-β production[35,36]. Indeed, native PAGE analysis showed LPS-induced formation of IRF-3 homodimers in wild-type cells. However, LPS-induced IRF-3 activation was abolished in cells from TRAM-deficient mice (Fig. 5c). In contrast, TRAM-deficient cells showed amounts of IRF-3 dimer formation similar to those of wild-type cells after poly(I:C) stimulation (Fig. 5d). As LPS-induced STAT-1 phosphorylation and IRF-3 activation are MyD88 independent, these data indicated that TLR4-mediated, but not TLR3-mediated, activation of the MyD88-independent signaling pathway was impaired in TRAM-deficient mice. In addition to IRF-3 activation, TLR4-mediated MyD88-independent signaling leads to late-phase NF-κB activation, which is evident in MyD88-deficient mice[16,21,22]. In contrast to wild-type macrophages, MyD88-deficient macrophages did not show NF-κB activation 10 min after LPS stimulation. However, we found NF-κB activation at 20 min or at later time points in MyD88-deficient macrophages[21,22]. Therefore, LPS-induced NF-κB activation is probably biphasic, and the early-phase NF-κB activation is MyD88

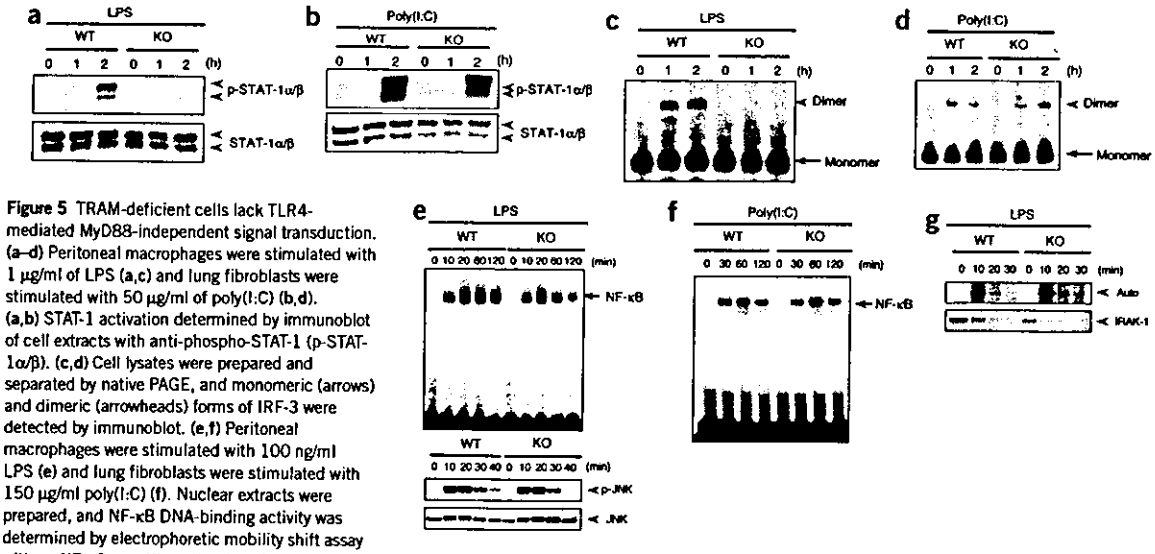

Figure 5 TRAM-deficient cells lack TLR4-mediated MyD88-independent signal transduction. (a–d) Peritoneal macrophages were stimulated with 1 μg/ml of LPS (a,c) and lung fibroblasts were stimulated with 50 μg/ml of poly(I:C) (b,d). (a,b) STAT-1 activation determined by immunoblot of cell extracts with anti-phospho-STAT-1 (p-STAT-1α/β). (c,d) Cell lysates were prepared and separated by native PAGE, and monomeric (arrows) and dimeric (arrowheads) forms of IRF-3 were detected by immunoblot. (e,f) Peritoneal macrophages were stimulated with 100 ng/ml LPS (e) and lung fibroblasts were stimulated with 150 μg/ml poly(I:C) (f). Nuclear extracts were prepared, and NF-κB DNA-binding activity was determined by electrophoretic mobility shift assay with an NF-κB-specific probe. (e) LPS-induced JNK activation was also determined by immunoblot with anti-phospho-JNK (bottom; p-JNK). (g) Lysates from macrophages stimulated with LPS were immunoprecipitated with anti-IRAK-1. The kinase activity of IRAK-1 was measured by *in vitro* kinase assay (top; Auto, auto-phosphorylation). The same lysates were blotted with anti-IRAK-1 (bottom). Stimulation times are above lanes. WT, wild-type; KO, TRAM-deficient.

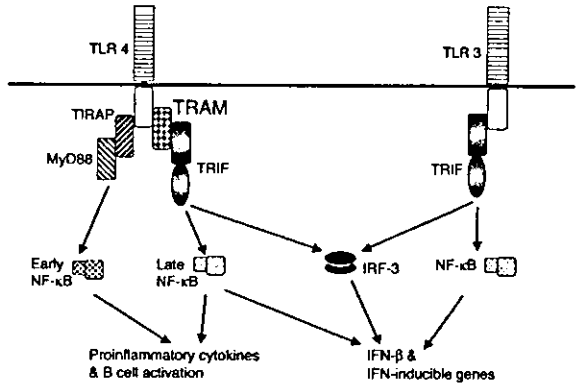

Figure 6 Participation of TIR domain–containing adaptor molecules in TLR3 and TLR4 signaling pathways. Four TIR domain–containing adaptors, MyD88, TIRAP, TRIF and TRAM, have been identified. MyD88 and TIRAP take part in TLR4-mediated proinflammatory cytokine production and B cell activation, but not in induction of the gene encoding IFN-β and IFN-inducible genes, the so-called MyD88-independent pathway[17,18,21,22]. TRIF is essential in the MyD88-independent pathway shared by TLR3 and TLR4 (refs. 25,26). TRAM specifically participates in the MyD88-independent pathway of TLR4, but not TLR3, signaling.

dependent, whereas the late-phase activation is MyD88 independent. Wild-type cells showed steady amounts of NF-κB activation even after 60 and 120 min of LPS stimulation. In TRAM-deficient cells, LPS-induced NF-κB activation occurred normally at early time points (10 and 20 min) but gradually faded at later time points (60 and 120 min), indicating that the late-phase NF-κB activation, which is characteristic of TLR4-mediated MyD88-independent signaling, was impaired in TRAM-deficient mice (Fig. 5e). In contrast, poly(I:C)-induced NF-κB activation was normal in TRAM-deficient cells (Fig. 5f). LPS stimulates activation of the kinase MAPK as well as NF-κB in a biphasic way. Even at 40 min after LPS stimulation, phosphorylated JNK was still present in wild-type cells. TRAM-deficient cells showed similar amounts of JNK activation at early time points (10 and 20 min). However, at 40 min, phosphorylated JNK was not detected in TRAM-deficient cells, indicating TRAM deficiency abolished the LPS-induced late-phase MAPK activation (Fig. 5e). Activation of the MyD88-dependent signaling pathway causes IRAK-1 activation, as indicated by autophosphorylation and degradation (Fig. 5g). TRAM-deficient macrophages showed LPS-induced IRAK-1 activation similar to that of wild-type cells, indicating that activation of TLR4-mediated MyD88-dependent signaling was intact in TRAM-deficient mice. These observations show that TRAM is essential specifically for the TLR4-mediated MyD88-independent signaling pathway (Fig. 6)

DISCUSSION

Here we analyzed the function of TRAM in the TLR–IL-1R signaling pathways *in vivo*. TRAM-deficient mice showed defects in cytokine production, splenocyte proliferation and up-regulation of surface molecules in response to the TLR4 ligand, but not to other TLR ligands. Furthermore, TLR4-mediated, but not TLR3-mediated, expression of the gene encoding IFN-β and IFN-inducible genes was inhibited in TRAM-deficient mice. In intracellular signal transduction, LPS-induced autophosphorylation of IRAK-1 and the early phase of NF-κB activation were intact in TRAM-deficient mice. However, we noted no activation of IRF-3 and a defect in the late phase of NF-κB activation in response to LPS, but not poly(I:C), in TRAM-deficient cells. Given that these events are features of the MyD88-independent signaling pathway, we propose that TRAM specifically mediates the MyD88-independent pathway of TLR4 signaling.

In TRAM-deficient mice, TLR4-mediated activation of the MyD88-dependent pathway, which is characterized by autophosphorylation of IRAK-1 and early-phase NF-κB activation, was similar to that of wild-type cells. However, TLR4-mediated production of proinflammatory cytokines was impaired. Similarly, TLR4-mediated production of proinflammatory cytokines was reduced in mice lacking TRIF, which is essential for TLR4- and TLR3-mediated MyD88-independent pathway. As MyD88-deficient mice showed similar phenotype, activation of the MyD88-independent pathway is apparently required for induction of proinflammatory cytokines[25,26]. Therefore, in TLR4 signaling, activation of both the MyD88-dependent and the MyD88-independent (TRAM–TRIF-dependent) pathways is required for proinflammatory cytokine production and B cell activation. However, in the signaling pathways of TLR2, TLR5 and TLR9, none of which activate the MyD88-independent pathway, activation of the MyD88-dependent pathway alone is sufficient to induce proinflammatory cytokines[8,34,37,38]. Therefore, at present, it remains unclear why TLR4 signaling requires activation of both the MyD88-dependent and the TRIF-dependent pathways to induce proinflammatory cytokines.

Individual TLRs provoke distinct cellular responses, as indicated by the presence of the MyD88-independent pathways in TLR3 and TLR4 signaling. Even in the MyD88-dependent pathways, different TLRs induce distinct types of gene expression. For example, TLR7 and TLR9 signaling induce type I IFN production[39], whereas TLR1, TLR2 and TLR6 ligands do not. Therefore, there must be some mechanisms to provide specificity to these signaling pathways. It is possible that other TIR domain–containing adaptor molecules like TIRAP are involved in MyD88-dependent TLR signals, such as TLR5, TLR7 and TLR9. In this case, the only remaining candidate is SARM[29,30]. The C-terminal portion of SARM contains the TIR domain in addition to the SAM and ARM domains. At present, neither *in vitro* nor *in vivo* analysis has shown whether SARM is involved in the TLR–IL-1R signaling pathways. Alternatively, it is possible that MyD88-dependent TLR5, TLR7, TLR9 or IL-1R signaling does not require such TIR domain–containing adaptor molecules in addition to MyD88. In this case, signaling molecules acting together with MyD88 should exist to generate the different cellular responses of these TLRs. These molecules may include the Pellino family of proteins, which was originally identified in *Drosophila melanogaster* as a molecule that is associated with Pelle, a Drosophila homolog of IRAK[40]. The Pellino family consists of three members at present. Pellino1 seems to activate NF-κB in the IL-1R signaling pathway through the integration of the IL-1R–IRAK1–IRAK4–TRAF6 complex[41]. Pellino2 and Pellino3 promote activation of the kinase JNK in the TLR-IL-1R signaling pathway[42–44]. Although the physiological functions of the Pellino family of proteins remain to be determined, it is possible that these proteins differentially take part in distinct TLR or IL-1R signaling pathways to diversify the cellular responses.

In summary, we have identified an essential adaptor that specifically mediates the MyD88-independent pathway of TLR4 signaling. Precise analysis of the involvement of TRAM in the LPS response of TRAM-deficient mice will provide a new insight into the relationship between TLR4-mediated MyD88-independent pathways and diseases caused by Gram-negative bacterial infection, such as septic shock.

METHODS

Plasmids. The ELAM-1 promoter–derived luciferase reporter plasmid (NF-κB luciferase reporter) was a gift from D.T. Golenbock (University of Massachusetts).

The mouse IFN-β promoter luciferase reporter was generated by PCR as described[45]. Flag-tagged human or mouse TRAM was cloned into the pEF-BOS vector. Expression vectors for TRIF and MyD88 were constructed as described[23].

Luciferase reporter assay. For luciferase assays, 293 cells were transiently transfected with reporter plasmids plus expression vectors. The luciferase activity of total cell lysates was measured with the Dual-Luciferase Reporter Assay System (Promega) as described[23]. The *Renilla* luciferase reporter gene (50 ng) was used as an internal control.

Generation of TRIF-deficient mice. The gene encoding TRAM was isolated from genomic DNA extracted from embryonic stem cells (E14.1) by PCR with TaKaRa LA Taq (TaKaRa). The targeting vector was constructed by replacement of a 1.0-kb fragment encoding the entire TRAM open reading frame with a neomycin-resistance gene cassette (neo^r), and a gene encoding herpes simplex virus thymidine kinase driven by a phosphoglycerate kinase promoter was inserted into the genomic fragment for negative selection (Fig. 1d). After the targeting vector was transfected into embryonic stem cells, G418 and gancyclovir double-resistant colonies were selected and screened by PCR and Southern blot analysis. Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progeny mice were intercrossed to obtain TRAM-deficient mice. TRAM-deficient mice and their wild-type littermates from these intercrosses were used for experiments. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University).

Reagents. R-848 was provided by the Pharmaceuticals and Biotechnology Laboratory of Japan Energy Corporation. CpG oligodeoxynucleotides were prepared as described[11]. LPS from *Salmonella minnesota* Re 595 (prepared by a phenol–chloroform–petroleum ether extraction procedure), PGN from *Staphylococcus aureus* and poly (I:C) were purchased from Sigma, Fluka and Amersham, respectively. Anti-phospho-JNK and anti-phospho-STAT-1 were purchased from Cell Signaling Technology. Anti-JNK1, anti-STAT-1 and anti-actin were from Santa Cruz Biotechnology. Polyclonal anti-TRAM was raised against amino acids 219–232 of mouse TRAM. This antibody specifically recognizes human and mouse TRAM *in vitro* (Supplementary Fig. 1 online). Polyclonal anti-IRF-3 and anti-IRAK-1 were as described[25]. Anti-IgM was purchased from Jackson ImmunoResearch Laboratory. Fluorescein isothiocyanate-labeled streptavidin (554060), phycoerythrin-conjugated anti-B220 (553090), biotin-conjugated anti-CD69 (553235), anti-CD86 (553690) and anti-I-A^b (06252D) were from Pharmingen.

Measurement of proinflammatory cytokine concentrations. Thioglycollate-elicited peritoneal macrophages were cultured in 96-well plates (5×10^4 cells per well) with PGN, LPS, R-848 or CpG DNA. The concentrations of TNF, IL-6 and IL-12 p40 in the culture supernatant were measured by ELISA according to the manufacturer's instructions (Genzyme for TNF-α and IL-12 p40; R&D Systems for IL-6).

Preparation of lung fibroblasts. Lungs from mice were excised, washed in PBS, cut into small pieces, agitated and digested enzymatically for 30 min at 37 °C. The digestion buffer (10 ml/lung) was composed of a 0.25% trypsin solution containing 400 nM EDTA. Ice-cold complete DMEM was added to the resulting cell suspension. After centrifugation (240*g* for 5 min), pellets were resuspended in complete medium, then cultured in dishes. Lung fibroblasts were used 10 d after excision for each experiment.

Electrophoretic mobility shift assay. Peritoneal macrophages and lung fibroblasts (1×10^6) were stimulated with 100 ng/ml of LPS and 50 μg/ml of poly(I:C), respectively. Nuclear extracts were purified from cells and incubated with a probe specific for NF-κB DNA-binding site, separated by electrophoresis and visualized by autoradiography as described[15].

Splenocyte proliferation assay. Splenocytes (1×10^5) were cultured in 96-well plates for 24 h with LPS, R-848 or CpG DNA. Samples were 'pulsed' with 1 μCi [^{3}H]thymidine for the last 12 h, and then ^{3}H uptake was measured in a β-scintillation counter (Packard).

Immunoblot analysis and *in vitro* kinase assay. Peritoneal macrophages (2×10^6), embryonic fibroblasts (1×10^6) and lung fibroblasts (1×10^6) were stimulated with 100 ng/ml of LPS, 10 ng/ml of IL-1β and 50 μg/ml of poly (I:C), respectively. The cells were then lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-Cl, pH 7.5, 1 mM EDTA and a protease inhibitor 'cocktail' (Roche Diagnostics). The cell lysates were separated by SDS-PAGE and transferred onto a polyvinylfluoride membrane (BioRad). The membrane was blotted with specific antibodies, and samples were visualized with an enhanced chemiluminescence system (Perkin Elmer Life Sciences). IRAK-1 activity in the cell lysates was measured by *in vitro* kinase assay as described[46].

Detection of TRAM protein by immunoprecipitation. For immunoprecipitation, cell lysates were precleared for 2 h with protein G–Sepharose (Amersham Pharmacia Biotech) and then incubated for 12 h with rotation at 4 °C with protein G–Sepharose containing 1.0 μg anti-TRAM. The immunoprecipitants were washed four times with lysis buffer, eluted by being boiled with Laemmli sample buffer and analyzed by immunoblot with anti-TRAM, as described above.

Flow cytometry. Splenocytes (2×10^6) were cultured with 50 μg/ml of poly(I:C), 10 μg/ml of LPS or 10 μg/ml of anti-IgM. After 36 h of culture, cells were collected and stained with phycoerythrin-conjugated anti-B220 and biotin-conjugated anti-CD69, anti-CD86 or anti-I-A^b followed by fluorescein isothiocyanate–conjugated streptavidin. Stained cells were analyzed by FACSCalibur with CellQuest software (Becton Dickinson).

Native PAGE assay. Lung fibroblasts (1×10^6) and peritoneal macrophages (5×10^6) were stimulated with 50 μg/ml of poly (I:C) and 1 μg/ml of LPS, respectively, and then lysed. Cell lysates in native PAGE sample buffer (62.5mM Tris-Cl, pH 6.8, 15% glycerol and 1% deoxycholate) were separated by native PAGE and then immunoblotted with anti-IRF-3 as described[46].

GenBank accession number. TRAM, AY232653.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Takeda, K., Kaisho, T. & Akira, S. Toll-like receptors. *Annu. Rev. Immunol.* **21**, 335–376 (2003).
2. Janeway, C.A. Jr. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).
3. Takeuchi, O. *et al.* Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* **169**, 10–14 (2002).
4. Alexopoulou, L. *et al.* Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in TLR1- and TLR2-deficient mice. *Nat. Med.* **8**, 878–884 (2002).
5. Takeuchi, O. *et al.* Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443–51 (1999).
6. Alexopoulou, L., Holt, A.C., Medzhitov, R. & Flavell, R.A. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732–738 (2001).
7. Hoshino, K. *et al.* Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* **162**, 3749–3752 (1999).
8. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085–2088 (1998).
9. Hayashi, F. *et al.* The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099–1103 (2001).
10. Takeuchi, O. *et al.* Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int.*

Immunol. **13**, 933–940 (2001).
11. Hemmi, H. *et al.* Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* **3**, 196–200 (2002).
12. Hemmi, H. *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745 (2000).
13. Wesche, H., Henzel, W.J., Shillinglaw, W., Li, S. & Cao, Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7**, 837–847 (1997).
14. Janssens, S. & Beyaert, R. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol. Cell.* **11**, 293–302 (2003).
15. Adachi, O. *et al.* Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**, 143–150 (1998).
16. Kawai, T., Adachi, O., Ogawa, T., Takeda, K. & Akira, S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**, 115–122 (1999).
17. Kawai, T. *et al.* Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* **167**, 5887–5894 (2001).
18. Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K. & Akira, S. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.* **166**, 5688–5694 (2001).
19. Horng, T., Barton, G.M. & Medzhitov, R. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat. Immunol.* **2**, 835–841 (2001).
20. Fitzgerald, K.A. *et al.* Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* **413**, 78–83 (2001).
21. Yamamoto, M. *et al.* Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* **420**, 324–329 (2002).
22. Horng, T., Barton, G.M., Flavell, R.A. & Medzhitov, R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* **420**, 329–333 (2002).
23. Yamamoto, M. *et al.* Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-β promoter in the Toll-like receptor signaling. *J. Immunol.* **169**, 6668–6672 (2002).
24. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. & Seya, T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nat. Immunol.* **4**, 161–167 (2003).
25. Yamamoto, M. *et al.* Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* **301**, 640–643 (2003).
26. Hoebe, K. *et al.* Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* **424**, 743–748 (2003).
27. Sharma, S. *et al.* Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**, 1148–1151 (2003).
28. Fitzgerald, K.A. *et al.* IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* **4**, 491–496 (2003).
29. Mink, M., Fogelgren, B., Olszewski, K., Maroy, P. & Csiszar, K. A novel human gene (SARM) at chromosome 17q11 encodes a protein with a SAM motif and structural similarity to Armadillo/β-catenin that is conserved in mouse, *Drosophila*, and *Caenorhabditis elegans*. *Genomics* **74**, 234–244 (2001).
30. O'Neill, L.A., Fitzgerald, K.A. & Bowie, A.G. The Toll-IL-1 receptor adaptor family grows to five members. *Trends. Immunol.* **24**, 286–290 (2003).
31. Bin, L.H., Xu, L.G. & Shu, H.B. TIRP, a novel Toll/interleukin-1 receptor (TIR) domain-containing adapter protein involved in TIR signaling. *J. Biol. Chem.* **278**, 24526–24532 (2003).
32. Kopydlowski, K.M. *et al.* Regulation of macrophage chemokine expression by lipopolysaccharide *in vitro* and *in vivo*. *J. Immunol.* **163**, 1537–1544 (1999).
33. Ohmori, Y. & Hamilton, T.A. Requirement for STAT-1 in LPS-induced gene expression in macrophages. *J. Leukoc. Biol.* **69**, 598–604 (2001).
34. Toshchakov, V. *et al.* TLR4, but not TLR2, mediates IFN-beta-induced STAT-1α/β-dependent gene expression in macrophages. *Nat. Immunol.* **3**, 392–398 (2002).
35. Doyle, S. *et al.* IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17**, 251–263 (2002).
36. Sakaguchi, S. *et al.* Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. *Biochem. Biophys. Res. Commun.* **306**, 860–866 (2003).
37. Hacker, H. *et al.* Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J. Exp. Med.* **192**, 595–600 (2000).
38. Schnare, M., Holt, A.C., Takeda, K., Akira, S. & Medzhitov, R. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol.* **10**, 1139–1142 (2000).
39. Vasilakos, J.P. *et al.* Adjuvant activities of immune response modifier R-848: comparison with CpG ODN. *Cell Immunol.* **204**, 64–74 (2000).
40. Grosshans, J., Schnorrer, F. & Nusslein-Volhard, C. Oligomerisation of Tube and Pelle leads to nuclear localisation of dorsal. *Mech. Dev.* **81**, 127–38 (1999).
41. Jiang, Z. *et al.* Pellino 1 is required for interleukin-1 (IL-1)-mediated signaling through its interaction with the IL-1 receptor-associated kinase 4 (IRAK4)-IRAK-tumor necrosis factor receptor-associated factor 6 (TRAF6) complex. *J. Biol. Chem.* **278**, 10952–10956 (2003).
42. Yu, K.Y. *et al.* Cutting edge: mouse pellino-2 modulates IL-1 and lipopolysaccharide signaling. *J. Immunol.* **169**, 4075–4078 (2002).
43. Jensen, L.E. & Whitehead, A.S. Pellino2 activates the mitogen activated protein kinase pathway. *FEBS Lett.* **545**, 199–202 (2003).
44. Jensen, L.E. & Whitehead, A.S. Pellino3, a novel member of the Pellino protein family, promotes activation of c-Jun and Elk-1 and may act as a scaffolding protein. *J. Immunol.* **171**, 1500–1506 (2003).
45. Sato, M. *et al.* Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity* **13**, 539–548 (2000).
46. Sato, S. *et al.* A variety of microbial components induce tolerance to lipopolysaccharide by differentially affecting MyD88-dependent and -independent pathways. *Int. Immunol.* **14**, 783–791 (2002).

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TOLL-LIKE RECEPTORS

Kiyoshi Takeda[1,2], Tsuneyasu Kaisho[1,2,3], and Shizuo Akira[1,2]

[1]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, [2]Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, [3]RIKEN Research Center for Allergy and Immunology, Suita Osaka 565-0871, Japan; email: sakira@biken.osaka-u.ac.jp, tkaisho@biken.osaka-u.ac.jp, ktakeda@biken.osaka-u.ac.jp

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■ **Abstract** The innate immune system in drosophila and mammals senses the invasion of microorganisms using the family of Toll receptors, stimulation of which initiates a range of host defense mechanisms. In drosophila antimicrobial responses rely on two signaling pathways: the Toll pathway and the IMD pathway. In mammals there are at least 10 members of the Toll-like receptor (TLR) family that recognize specific components conserved among microorganisms. Activation of the TLRs leads not only to the induction of inflammatory responses but also to the development of antigen-specific adaptive immunity. The TLR-induced inflammatory response is dependent on a common signaling pathway that is mediated by the adaptor molecule MyD88. However, there is evidence for additional pathways that mediate TLR ligand-specific biological responses.

INTRODUCTION

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and non-self. In mammals the immune system can be divided into two branches: "innate immunity" and "adaptive immunity." Adaptive immunity detects non-self through recognition of peptide antigens using antigen receptors expressed on the surface of B and T cells. In order to respond to a wide range of potential antigens, B and T cells rearrange their immunoglobulin and T cell receptor genes to generate over 10^{11} different species of antigen receptors. Engagement of antigen receptors by the cognate antigen triggers clonal expansion of the lymphocyte and further production of antigen-specific antibodies. This highly sophisticated system is observed only in vertebrates and is a potent defense against microbial infection. In contrast, the innate immune system, which was first described over a century ago, is phylogenetically conserved and is present in almost all multicellular organisms (1). Whereas the system of adaptive immunity

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has been the subject of considerable study in the past century, innate immunity has been less well appreciated. Therefore, the mechanism by which innate immunity recognizes non-self remained unknown until quite recently. However, the recent identification of Toll-like receptors in mammals has made immunologists aware that innate immunity plays an important role in the detection of invading pathogens. Recent evidence shows that Toll-like receptors recognize specific patterns of microbial components, especially those from pathogens, and regulates the activation of both innate and adaptive immunity. In this review we focus on recent progress regarding the functions of Toll-like receptors and their signaling pathways.

TOLL IN DROSOPHILA

The Toll Pathway

The involvement of the Toll receptors in innate immunity was first described in drosophila. Drosophila Toll was originally identified as a transmembrane receptor required for the establishment of dorso-ventral polarity in the developing embryo (2). Stimulation of Toll by the secreted Spätzle factor, a ligand of Toll, activates the cytoplasmic serine/threonine kinase Pelle via the adaptor protein Tube. Activation of Pelle promotes degradation of the ankyrin-repeat protein Cactus, which associates with the Rel-type transcription factor Dorsal in the cytoplasm. Once Cactus is degraded in response to the Toll-mediated signal, Dorsal is free to translocate to the nucleus, where it regulates transcription of specific target genes (3). The signaling pathway of drosophila Toll shows remarkable similarity to the mammalian IL-1 pathway, which leads to activation of NF-κB, a transcription factor responsible for many aspects of inflammatory and immune responses. Indeed, the cytoplasmic domains of drosophila Toll and the mammalian IL-1 receptor are highly conserved and are referred to as the Toll/IL-1 receptor (TIR) domain. Based on this similarity, it was proposed that the Toll-mediated pathway might be involved in regulating immune responses (3). This was clearly demonstrated in a study of mutant flies lacking individual components of the Toll-mediated pathway, i.e., Toll, Spätzle, Tube, or Pelle (4). Each mutant fly was highly sensitive to fungal infection owing to a lack of expression of the antifungal peptide Drosomycin. In drosophila two additional Rel-type transcription factors, Dorsal-type immune factor, and Relish, have been identified in addition to Dorsal. Dorsal-type immune factor is mainly involved in the induction of antifungal peptide genes in adult flies, whereas Dorsal is involved in dorso-ventral patterning in the embryo (5, 6). Relish regulates the induction of peptides active against Gram-negative bacteria (7). A recent study indicated that the Toll pathway is required for resistance to Gram-positive bacterial infections in addition to fungal infections (8). Indeed, infection with either Gram-positive bacteria or fungi induced Toll-dependent expression of the antifungal peptide Drosomycin.

Tube is an adaptor that functions downstream of Toll and upstream of Pelle. Although Tube and Pelle have been shown to interact via conserved death domains

(9, 10), no direct interaction has been demonstrated between Tube and Toll. However, the protein DmMyD88 appears to function as an adaptor linking Toll and Pelle; the TIR domain of DmMyD88 associates with the TIR domain of Toll, and the death domain of DmMyD88 associates with the death domain of Pelle (11, 12). DmMyD88 mutant flies are highly sensitive to fungal infection, suggesting that DmMyD88 is an essential component of the Toll pathway in drosophila. However, the functional relation between Tube and DmMyD88 remains unclear.

The protein Spätzle is secreted as a precursor form that is cleaved to its active form by a serine protease in response to immune challenge. The cleaved Spätzle then activates Toll. Mutant flies with a loss-of-function mutation in the gene encoding the serine protease inhibitor Spn43Ac exhibit constitutive expression of cleaved Spätzle and, consequently, constitutive expression of Drosomycin (13). These data demonstrate that Toll is indirectly activated by Spätzle, rather than directly by microbial components. The precise mechanism by which Toll is activated in response to microbial infection is not well understood, but a recent genetic study has provided some possible clues. An ethyl-methyl-sulfonate–induced mutation of the *semmelweis* (*seml*) gene was shown to cause impaired expression of Drosomycin in response to infection by Gram-positive bacteria but not fungi (14). The gene responsible for the mutation was analyzed and found to encode PGRP-SA, the peptidoglycan recognition protein. PGRP-SA recognizes peptidoglycans that are abundant in Gram-positive bacterial cell walls (15). Thus, infection by Gram-positive bacteria is detected by PGRP-SA, which in turn activates the Toll-mediated pathway. A factor that is involved in the detection of fungal infection and activation of the Toll-mediated pathway has recently been identified. Ethyl-methyl-sulfonate mutagenesis of drosophila produced a mutant with impaired activation of the Toll pathway in response to fungal infection but not to Gram-positive bacterial infection (16). This mutation was localized to the *Persephone* gene, which encodes a serine protease but possesses no obvious microbial pattern recognition domain (Figure 1). Most likely, there is a molecule upstream of Persephone that detects fungal infection. Identification of this molecule may provide new insights into the mechanisms of microbial recognition in drosophila.

IMD Pathway

The immune response against Gram-negative bacteria is mediated by a distinct pathway first identified by a mutation in the *immune deficiency* (*imd*) gene of drosophila (17). *Imd* mutant flies are highly susceptible to infection by Gram-negative bacteria but not to fungi, whereas Toll mutants are highly susceptible to fungi but not to Gram-negative bacteria. The *imd* gene encodes an adaptor protein containing a death domain with similarity to the mammalian receptor interacting protein (18). Genetic studies have identified several molecules involved in the IMD pathway that are involved in the response against Gram-negative bacteria. These include DmIKKβ, DmIKKγ, dTAK1, and Relish (7, 19–22). Fruitflies with mutations in these genes are defective in expression of the antibacterial peptide

Diptericin and highly susceptible to Gram-negative bacterial infection. No receptor involved in the IMD pathway has been identified. Mutant flies lacking 18-wheeler, a member of the Toll family, are susceptible to Gram-negative bacterial infection, but expression of *Diptericin* is normal in these mutants (23, 24). Moreover, there are nine Toll family members in drosophila, but none of them has been shown to induce expression of *Diptericin* (25). These results indicate the existence of a non-Toll-related receptor that initiates signaling in the IMD pathway. Indeed, a member of the PGRP family, PGRP-LC, has been implicated in the activation of the IMD pathway, because induction of *Diptericin* in response to Gram-negative bacterial infection was shown to be defective in PGRP-LC mutant flies (26–28). Unlike PGRP-SA, PGRP-LC is a transmembrane protein (15). Although it remains unclear whether the intracellular portion of PGRP-LC possesses a domain required for activation of signaling cascades, it is possible that this protein may act as a receptor linking the recognition of Gram-negative bacteria to the activation of the IMD pathway (Figure 1). PGRPs in drosophila consist of a large family containing 12 members (15) and thus could be involved in sensing a variety of different microbes in drosophila.

One component of the IMD pathway, Relish, is activated by a cleavage into two domains: the DNA-binding Rel homology domain and the inhibitory ankyrin repeat domain. Although the mechanism by which Relish is cleaved is unclear, it has been suggested that Dredd, a homologue of mammalian caspase-8, may be somehow involved. Dredd mutant flies are defective in cleavage of Relish, and are very susceptible to infection by Gram-negative bacteria (29–31). Dredd associates with drosophila Fas associating death domain (dFADD), a homologue of mammalian FADD (12, 32). In mammals the FADD/caspase8-dependent pathway is activated as a result of signaling from the type I TNF receptor (TNF-RI) and mediates induction of apoptosis. Overexpression of IMD, a homologue of mammalian receptor interacting protein that associates with TNF-RI, induces apoptosis in drosophila, whereas *imd* mutant flies are resistant to UV-induced apoptosis (18). Thus, the IMD pathway is presumably involved in the induction of apoptosis as well as the response against Gram-negative bacteria. This also indicates that IMD may act upstream of dFADD-Dredd, similar to receptor interacting protein's acting upstream of FADD-casapase-8 in the mammalian TNF-RI-mediated signal pathway.

TOLL-LIKE RECEPTORS IN MAMMALS

A year after the discovery of the role of the drosophila Toll in the host defense against fungal infection, a mammalian homologue of the drosophila Toll was identified (33). Subsequently, a family of proteins structurally related to drosophila Toll was identified, collectively referred to as the Toll-like receptors (TLRs). The TLR family is known to consist of 10 members (TLR1-TLR10), and no doubt more will be found in the future (33–38). The chromosomal location of each human TLR

gene has been determined. *TLR1* and *TLR6* map very close to 4p14 (34, 35); *TLR2* and *TLR3* map to 4q32 and 4q35, respectively; *TLR4* and *TLR5* map to 9q32-33 and 1q33.3, respectively (34). *TLR7* and *TLR8* are located in tandem in Xp22, whereas *TLR9* maps to 3p21.3 (36, 38).

TLR family members are characterized structurally by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a TIR domain in their intracellular domain. A comparison of the amino acid sequences of the human TLRs reveals that members of the TLR family can be divided into five subfamilies: the TLR3, TLR4, TLR5, TLR2 and TLR9 subfamilies (Figure 2). The TLR2 subfamily is composed of TLR1, TLR2, TLR6, and TLR10; the TLR9 subfamily is composed of TLR7, TLR8, and TLR9. In the TLR2 subfamily TLR1 and TLR6 are highly similar proteins and exhibit 69.3% identity in overall amino acid sequence, but the TIR domains of both receptors are highly conserved, with over 90% identity (35). Because *TLR1* and *TLR6* have similar genomic structures, consisting of one exon, and are located in tandem in the same chromosome, they may be the products of an evolutionary duplication. Division of TLRs into five subfamilies is also based on genomic structure. The *TLR2* gene has two exons, but all of the coding sequences are contained within, exon 2. In contrast, the TLR9 subfamily members including TLR7, TLR8, and TLR9 are encoded by two exons (36, 38). The genes for TLR7 and TLR8 show 42.3% identity and 72.7% similarity in their amino acid sequences, have similar genomic structures, and are located close to each other on the X chromosome (36, 38). The *TLR4* and *TLR5* genes have four and five exons,

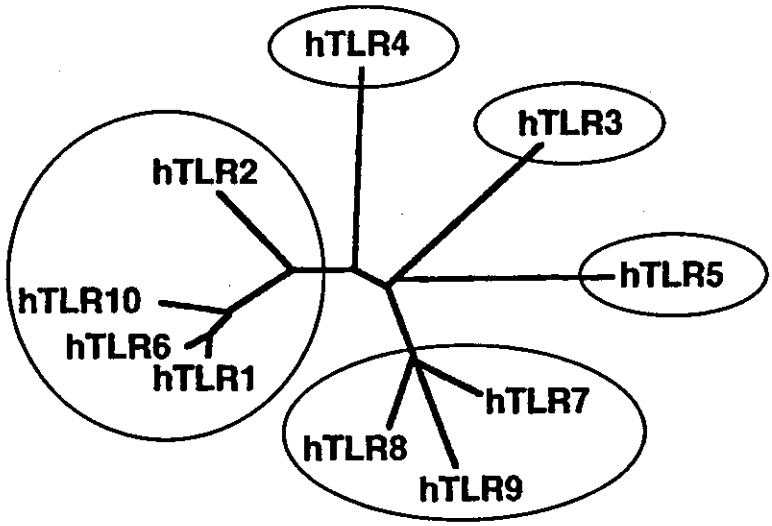

Figure 2 Phylogenetic tree of human Toll-like receptors (TLRs). The phylogenetic tree was derived from an alignment of the amino acid sequences for the human TLR members using the neighbor-joining method.

respectively. TLR3 has a unique structure among the TLRs in that it has five exons and the protein is encoded by exons 2 through 5. This is in contrast to all of the other TLRs, which are encoded by only one or two exons.

ROLES OF TLRS IN RECOGNITION OF MICROBIAL COMPONENTS

Ectopic overexpression of TLR4, the first mammalian TLR identified, was shown to cause induction of the genes for several inflammatory cytokines and costimulatory molecules (33). Therefore, it was anticipated that the TLRs might be involved in immune responses, especially in the activation of innate immunity. In 1998 TLR4 was shown to be involved in the recognition of lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria. Subsequently, other members of the TLR family have been shown to be essential for the recognition of a range of microbial components (Table 1). The structural similarity of TLRs seems to reflect their common function in the recognition of microbial components.

TLR4

TLR4 RECOGNIZES LIPOPOLYSACCHARIDE Two mouse strains, C3H/HeJ and C57BL10/ScCr, have long been known to be hypo-responsive to LPS. Two independent groups searching for the genes responsible for this hyporesponsiveness identified mutations in *Tlr4* (39, 40). The C3H/HeJ mouse strain has a point mutation in the intracellular region of the *Tlr4* gene leading to the replacement of a highly conserved proline with histidine. This mutation results in the generation of a dominant negative allele, defects in TLR4-mediated signaling, and consequent suppression of the response to LPS (41). Another LPS hypo-responsive strain, C57BL10/ScCr, has a null mutation in the *Tlr4* gene (39, 40). TLR4-deficient mice generated by gene targeting are also hypo-responsive to LPS, confirming that TLR4 is an essential receptor for the recognition of LPS (41).

Recognition of LPS requires other molecules in addition to TLR4. LPS binds to LPS-binding protein, present in the serum, and this LPS–LPS-binding protein complex is subsequently recognized by CD14, a glycosylphosphatidylinositol-anchored molecule preferentially expressed in monocytes/macrophages and neutrophils. LPS stimulation is followed by increased physical proximity between CD14 and TLR4, suggesting that CD14 and TLR4 may interact in LPS signaling (42, 43). MD-2 was identified as a molecule that associates with the extracellular portion of TLR4 and enhances LPS responsiveness (44, 45). Chinese hamster ovary cell lines that are hypo-responsive to LPS have mutations in the *MD-2* gene (46). Generation of MD-2-deficient mice demonstrated its essential role in the response to LPS. Macrophages, dendritic cells, and B cells from MD-2-deficient mice display severely impaired responses to LPS. Furthermore, MD-2-deficient mice are resistant to LPS-induced endotoxin shock, similar to TLR4-deficient mice (47). MD-2 associates with TLR4 in the endoplasmic reticulum/cis Golgi and then the

TABLE 1 Toll-like receptors and their ligands

TLR family	Ligands (origin)
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria)
	Soluble factors (Neisseria meningitides)
TLR2	Lipoprotein/lipopeptides (a variety of pathogens)
	Peptidoglycan (Gram-positive bacteria)
	Lipoteichoic acid (Gram-positive bacteria)
	Lipoarabinomannan (mycobacteria)
	A phenol-soluble modulin (Staphylococcus epidermidis)
	Glycoinositolphospholipids (Trypanosoma Cruzi)
	Glycolipids (Treponema maltophilum)
	Porins (Neisseria)
	Zymosan (fungi)
	Atypical LPS (Leptospira interrogans)
	Atypical LPS (Porphyromonas gingivalis)
	HSP70 (host)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria)
	Taxol (plant)
	Fusion protein (RSV)
	Envelope proteins (MMTV)
	HSP60 (Chlamydia pneumoniae)
	HSP60 (host)
	HSP70 (host)
	Type III repeat extra domain A of fibronectin (host)
	Oligosaccharides of hyaluronic acid (host)
	Polysaccharide fragments of heparan sulfate (host)
	Fibrinogen (host)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopeptides (mycoplasma)
TLR7	Imidazoquinoline (synthetic compounds)
	Loxoribine (synthetic compounds)
	Bropirimine (synthetic compounds)
TLR8	?
TLR9	CpG DNA (bacteria)
TLR10	?

TLR4/MD-2 complex moves to the cell surface, where excess MD-2 is secreted (48). Whereas TLR4 normally resides on the cell surface in wild-type cells, it is found in the Golgi apparatus in cells deficient for MD-2, indicating that MD-2 is essential for the intracellular distribution of TLR4 (47). Another cell-surface protein, RP105, is also involved in the recognition of LPS. RP105 contains an LRR domain that is structurally related to those found in the extracellular portion of the TLRs

and is preferentially expressed on the surface of B cells (49). B cells from RP105–deficient mice show a severely reduced response to LPS. RP105 functionally associates with TLR4 to recognize LPS (50). Thus, several components are implicated in the recognition of LPS, indicating that the functional LPS receptor forms a large complex.

OTHER TLR4 LIGANDS In addition to LPS, TLR4 recognizes several other ligands. Taxol is a product of the Pacific yew (*Taxus brevifolia*) and exhibits potent antitumor activity in humans. The antimitotic action of Taxol is due to its ability to bind and stabilize microtubules, which prevents proper cell division during mitosis. Taxol possesses similar immunostimulatory activities to those of LPS in mice but not in humans. Mouse TLR4 and MD-2 mediate the LPS-mimetic activity of Taxol (51–53). TLR4 and CD14 recognize the fusion protein of respiratory syncytical virus (54, 55). Accordingly, C3H/HeJ and C57BL/10ScNCr mice, which are mutated for TLR4, exhibited a reduced inflammatory response against and impaired clearance of respiratory syncytical virus. Activation of B cells by murine retroviruses such as mouse mammary tumor virus is dependent on TLR4. The envelope proteins of mouse mammary tumor virus and Moloney murine leukemia virus were reported to co-immunoprecipitate with TLR4 (56). Thus, TLR4 is presumably involved in the recognition of a certain group of viruses.

TLR4 seems to recognize some endogenous ligands as well. Heat shock proteins are highly conserved among organisms ranging from bacteria to mammals. A wide variety of stressful conditions such as heat shock, ultraviolet radiation, and viral and bacterial infection induce the increased synthesis of heat shock proteins. The primary functions of heat shock proteins are to chaperone nascent or aberrantly folded proteins. In addition, heat shock proteins activate macrophages and dendritic cells to secrete proinflammatory cytokines and to express costimulatory molecules. Thus, heat shock proteins may be representative of a type of endogenous "danger signal," i.e., molecules or molecular structures that are released or produced by cells undergoing stress or abnormal cell death (necrosis). These signals are recognized by macrophages and dendritic cells and thereby initiate immune responses (57).

The ability of heat shock protein to activate the immune cells is best documented for the heat shock protein HSP60. The immuno-stimulatory activity of HSP60 is mediated by TLR4 (58, 59). For example, HSP60 has been implicated in inflammation accompanying atherosclerosis, development of which is associated with chronic infection by *Chlamydia pneumoniae*. HSP60 derived from *Chlamydia pneumoniae* (cHSP60) colocalizes with macrophages in the atheromatous lesion and induces an inflammatory response. Therefore, cHSP60 is thought to be one of the factors causing atherosclerosis in chronic Chlamydial infection. cHSP60 also activates vascular smooth muscle cells and macrophages through TLR4 (60, 61). Mice defective for TLR4 show defective production of inflammatory cytokines in response to HSP70 as well as HSP60 (62–64). Thus, TLR4 seems to be responsible for the inflammatory responses elicited by heat shock proteins. However,

both TLR2 and TLR4 are required for recognition of HSP70 (63, 64). CD91 (α-macroglobulin receptor) has been identified as a receptor for several heat shock proteins, including HSP70 (65). Furthermore, HSP60 binds to macrophages from TLR4-deficient C57BL/10ScCr mice, despite the fact that no HSP60-induced production of inflammatory cytokines is observed (66). These data suggest that TLR4 is not directly involved in the recognition of heat shock proteins.

Extracellular matrix components, including fibronectin, hyaluronic acid, and heparan sulfate, are produced in response to tissue injury and play important roles in tissue remodeling, such as containing the agent of injury, closing the wound, and completing the healing. The type III repeat extra domain A of fibronectin has immuno-stimulatory activities similar to those provoked by LPS. This response to extra domain A of fibronectin is mediated by TLR4 (67). In addition, low molecular weight oligosaccharides of hyaluronic acid have been reported to be potent activators of dendritic cells, and activation of dendritic cells by hyaluronic acid is mediated by TLR4 (68). Furthermore, polysaccharide fragments of heparan sulfate have been reported to induce maturation of dendritic cells via TLR4 (69). Extravascular fibrin deposits are an early and persistent hallmark of inflammation accompanying injury, infection, and immune disorders. Fibrin is generated from plasma-derived fibrinogen, which escapes the vasculature in response to endothelial cell retraction at sites of inflammation. The capacity of fibrinogen to induce the production of chemokines from macrophages is elicited through recognition by TLR4 (70). Thus, TLR4 is presumably involved in several aspects of the inflammatory response by recognizing endogenous ligands produced during inflammation, even in the absence of infection. However, it should be noted that all of these endogenous TLR4 ligands activate immune cells only at very high concentrations, which is in sharp contrast to the low concentrations required for lipopolysaccharide (LPS). Therefore, there remains the possibility that these endogenous ligands might be contaminated with a true TLR4 ligand such as LPS.

One intriguing question is whether TLR4 recognizes its ligands directly or not. Some groups have proposed that recognition of LPS by TLR4 involves direct binding, while others have suggested that LPS binds to MD-2, and this complex somehow stimulates TLR4 (71–74). Species-specific recognition of different ligands provides one kind of genetic evidence for direct interaction. For example, mouse but not human cells recognize Taxol, and this species-specific recognition is conferred by MD-2 (53). Another group showed that human but not murine TLR4 recognizes the highly acylated LPS from *Pseudomonas aeruginosa* (75).

TLR4-INDEPENDENT RECOGNITION OF LIPOPOLYSACCHARIDE TLR4 has now been established as an essential component in the recognition of LPS. However, several reports have indicated that LPS can also be recognized independently of TLR4. A study using affinity chromatography, peptide mass fingerprinting, and fluorescence resonance energy transfer identified four molecules on the cell surface that bind LPS. These are HSP70, HSP90, chemokine receptor 4 (CXCR4), and growth differentiation factor 5 (76).

LPS is rapidly delivered into the cytoplasm after binding to the cell surface. This intracellular movement appears to be necessary for certain cellular responses, since agents that block vesicular transport such as wortmannin or cytochalasin D block the integrin-mediated adhesion of neutrophils in response to LPS (77). This suggests that LPS may be recognized in the cytoplasm as well as on the cell surface. A candidate molecule that confers the intracellular recognition of LPS is Nod1. Nod1 was originally identified as a molecule that is structurally related to the apoptosis regulator, Apaf-1, which contains the caspase-recruitment domain and the nucleotide-binding oligomerization domain. Nod1 possesses an N-terminal caspase-recruitment domain linked to a nucleotide-binding domain and a C-terminal LRR domain. Unlike Apaf-1, Nod1 induces activation of NF-κB (78). Nod1 mediates activation of NF-κB in response to LPS and cell-invasive *Shigella flexneri*, indicating that Nod1 is a cytoplasmic receptor for LPS (79, 80). These findings suggest that the Nod family of proteins is involved in inflammatory responses, possibly through the recognition of LPS in the cytoplasm. Nod2, a molecule in the same family as Nod1 and Apaf-1, also confers LPS-induced activation of NF-κB. Furthermore, frameshift and missense mutations in *NOD2* are associated with susceptibility to Crohn's disease (81, 82). However, the mutations found in these patients are restricted to the LRR domain, which presumably recognizes LPS, and the mutant NOD2 protein is defective in LPS-induced NF-κB activation (81, 82). Therefore, it remains unclear exactly how mutation of *NOD2* may be associated with susceptibility to Crohn's disease.

TLR2, TLR1, and TLR6

TLR2 RECOGNIZES A VARIETY OF MICROBIAL COMPONENTS TLR2 recognizes components from a variety of microorganisms. These include lipoproteins from pathogens such as Gram-negative bacteria, Mycoplasma and spirochetes (83–87), peptidoglycan and lipoteichoic acid from Gram-positive bacteria (88–91), lipoarabinomannan from mycobacteria (90–93), glycoinositolphospholipids from *Trypanosoma Cruzi* (94), a phenol-soluble modulin from *Staphylococcus epidermidis* (95), zymosan from fungi (96), glycolipids from *Treponema maltophilum* (97), and porins that constitute the outer membrane of *Neisseria* (98). Analysis of TLR2-deficient mice showed that TLR2 is critical to the recognition of peptidoglycan and lipoproteins (99, 100). Accordingly, TLR2-deficient mice showed higher susceptibility to infection by the Gram-positive bacteria *S. aureus* than wild-type mice (101). Another TLR2-deficient mouse strain showed defective clearance of spirochetes after infection by *Borrelia burgdorferi* and unresponsiveness to *B. burgdorferi* lipoproteins (102). Furthermore, TLR2 recognizes several atypical types of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*, in contrast to TLR4, which recognizes LPSs from enterobacteria such as *Escherichia coli* and *Salmonella* spp. (103, 104). The properties of the atypical LPSs recognized by TLR2 differ structurally and functionally from the enterobacteria LPS recognized by TLR4. In particular, the two types of LPS differ structurally in the number of

acyl chains in the lipid A component (105). TLR2 and TLR4 may differentially recognize these structural variations in LPS. However, as with many of these studies, it remains possible that very small amounts of contaminating TLR2 ligand in the LPS preparations might obscure some of these results.

TLR2 COOPERATES WITH TLR1 AND TLR6 One aspect of TLR2 ligand recognition involves cooperation with other TLR family members, in particular TLR6 and TLR1, which confer discrimination among different microbial components. The role of TLR6 was analyzed by introducing a dominant negative form into the RAW264.7 macrophage cell line. Peptidoglycan and secreted modulin from *S. aureus* are TLR2 ligands that induce TNF-α production in RAW264.7 cells, but these responses are suppressed by expression of dominant negative TLR6 (95, 106). TLR2 and TLR6 co-immunoprecipitate, suggesting that they physically interact in the cell (106). Analysis of TLR6-deficient mice further demonstrated that TLR6 functionally cooperates with TLR2 to recognize microbial lipopeptides (107). For example, bacterial lipopeptides have a NH_2-terminal cysteine residue that is triacylated, in contrast to mycoplasmal macrophage-activating lipopeptides 2 (MALP-2) which are only diacylated. Macrophages from TLR6-deficient mice did not show any inflammatory response to MALP-2, whereas these cells responded normally to bacterial lipopeptides. Macrophages from TLR2-deficient mice showed no response to either type of lipopeptide. Reconstitution experiments in TLR2/TLR6 doubly deficient embryonic fibroblasts demonstrated that both TLR2 and TLR6 are required for the response to MALP-2. Thus, TLR6 functionally associates with TLR2 to confer specific recognition of the subtle differences between triacyl and diacyl lipopeptides.

TLR1 has also been reported to functionally associate with TLR2. Cotransfection of TLR1 and TLR2 into HeLa cells confers responsiveness to soluble factors released from *Neisseria meningitidis* (108). Analysis of TLR1-deficient mice has demonstrated the importance of TLR1 in the recognition of triacyl lipopeptides (109). Macrophages from TLR1-deficient mice showed impaired production of inflammatory cytokines in response to several kinds of triacyl lipopeptides and lipoproteins from mycobacteria. When a range of triacyl lipopeptides with different lengths of fatty acid chains at their N-terminal cysteines was tested on cells from TLR1-deficient mice, the response to lipopeptides with an N-palmytoyl-S-dilauryl cysteine residue was found to be the most impaired. Although this impairment was not complete, this study suggests that TLR1 is responsible for recognizing subtle differences among the lipid moieties of lipopeptides. TLR1 is highly homologous to TLR6. Therefore, TLR6 may in some cases compensate for a deficiency in TLR1, or other TLRs, in the recognition of triacyl lipopeptides. Involvement of TLR1 in the recognition of the outer surface lipoprotein of *B. burgdorferi* was also shown (110).

Thus, TLR2 has been shown to functionally associate with several TLRs, at least TLR1 and TLR6, and it recognizes a wide variety of microbial components. It is unknown whether dimerization of TLR2 with other TLRs occurs constitutively