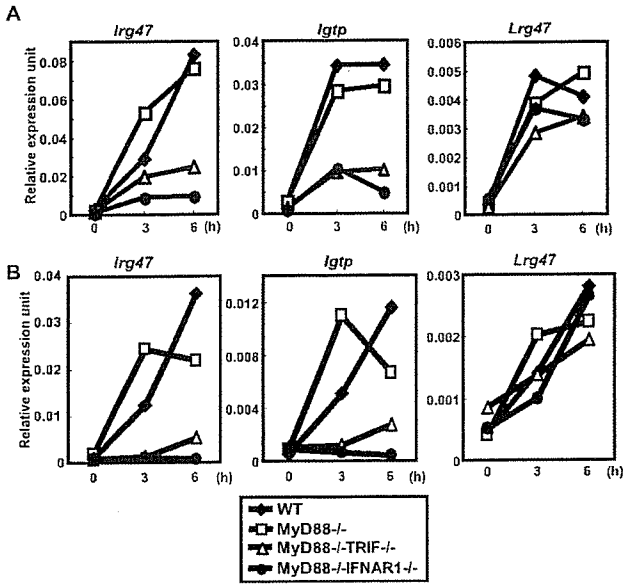

FIGURE 7. Impaired expression of IRG47 in *T. cruzi*-infected MyD88$^{-/-}$TRIF$^{-/-}$ mice. Bone marrow-derived macrophages (*A*) or DCs (*B*) from WT, MyD88$^{-/-}$, MyD88$^{-/-}$TRIF$^{-/-}$, or MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were infected with *T. cruzi* for 3 or 6 h. Next, total RNA was extracted and analyzed for the expressions of *Irg47*, *Igtp*, *and Lrg47* by quantitative real-time RT-PCR. Data are shown as the relative mRNA levels normalized to the corresponding EF-1α mRNA level.

and IRG47, and inducibly expressed GTPase (IGTP), has been shown to be induced through activation of TLR and IFN signaling pathways during infection with intracellular pathogens (27, 28). Therefore, we analyzed the expression levels of these p47 GTPases in *T. cruzi*-infected DCs and macrophages. Bone marrow-derived macrophages or DCs from WT, MyD88$^{-/-}$, MyD88$^{-/-}$TRIF$^{-/-}$, and MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were infected with *T. cruzi* for 3 or 6 h, and the expression of LRG47, IRG47, and IGTP mRNAs was analyzed (Fig. 7, *A* and *B*). In WT and MyD88$^{-/-}$ macrophages and DCs, *T. cruzi* infection resulted in robust mRNA expressions of all these p47 GTPases. Even in MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ cells, almost normal *T. cruzi*-induced expression of LRG47 mRNA was observed. However, *T. cruzi*-induced expression of IRG47 and IGTP mRNAs was severely impaired in MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages and DCs. Although IGTP was previously shown to have a minor role in *T. cruzi* infection, the involvement of IRG47 in *T. cruzi* infection is less well defined (29). Therefore, we next analyzed whether IRG47 is responsible for antitrypanosomal responses in the absence of MyD88. To complete this analysis, siRNA-mediated knockdown of IRG47 was performed in MyD88$^{-/-}$ macrophages. We transfected an IRG47 or control siRNA into bone marrow-derived macrophages and extracted the total RNA after 18 h for analysis of the IRG47 expression (Fig. 8*A*). Introduction of the IRG47 siRNA into bone marrow-derived macrophages from MyD88$^{-/-}$ mice resulted in an effective (81%) reduction in IRG47 mRNA expression. MyD88$^{-/-}$ macrophages transfected with the IRG47 or control siRNA were further infected with *T. cruzi*, and the intracellular parasites were visualized and counted (Fig. 8, *B* and *C*). In MyD88$^{-/-}$ macrophages, siRNA-mediated knockdown of IRG47 led to increased numbers of intracellular *T. cruzi*. These results indicate that IRG47 is involved in resistance to *T. cruzi* infection in innate immune cells.

Discussion

In the present study, we analyzed innate immune responses to the intracellular protozoan parasite *T. cruzi* using MyD88$^{-/-}$TRIF$^{-/-}$ mice, in which TLR-dependent activation of innate immunity is not induced. Macrophages and DCs derived from MyD88$^{-/-}$TRIF$^{-/-}$ mice showed impaired clearance of *T. cruzi*. Analysis of the gene expression profiles of *T. cruzi*-infected MyD88$^{-/-}$TRIF$^{-/-}$ DCs revealed that IFN-β was induced in a TRIF-dependent manner during *T. cruzi* infection, whereas analyses with an anti-IFN-β neutralizing Ab and MyD88$^{-/-}$IFNAR1$^{-/-}$ cells demonstrated that IFN-β mediated antitrypanosomal innate immune responses. Furthermore, both MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were highly sensitive to in vivo *T. cruzi* infection. These findings indicate that MyD88-dependent induction of proinflammatory cytokines and TRIF-dependent induction of IFN-β both contribute to innate immune responses to *T. cruzi* infection. We further showed that the p47 GTPase IRG47 is responsible for the resistance to *T. cruzi* infection in MyD88$^{-/-}$ macrophages.

Type I IFNs are well-known cytokines that exhibit antiviral activities (30). However, a large body of evidence has demonstrated

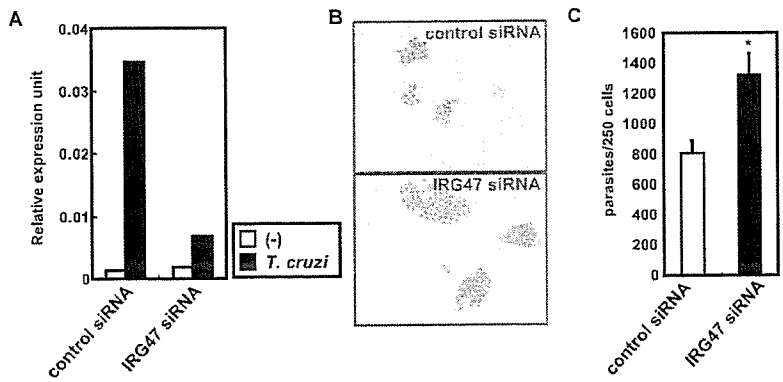

FIGURE 8. IRG47 mediates antitrypanosomal activity in MyD88$^{-/-}$ mice. *A*, Bone marrow-derived macrophages were transfected with IRG47 or control siRNA and cultured for 18 h. The cells were then infected with *T. cruzi* for 6 h and analyzed for the expression of IRG47 mRNA by quantitative real-time RT-PCR. Data are shown as the relative mRNA levels normalized to the corresponding EF-1α mRNA level. *B* and *C*, Bone marrow-derived macrophages transfected with an IRG47 or control siRNA were infected with *T. cruzi*, washed, and cultured for 48 h. The cells were then fixed, stained, and analyzed by microscopy. Representative stained cells from three independent experiments are shown. Magnification, ×400. Intracellular parasites were counted, and the data represent the mean ± SD of the number of parasites per 250 macrophages. *, $p < 0.02$ compared with control siRNA-transfected cells.

that type I IFNs are also induced by nonviral pathogens, such as bacteria, mycobacteria, and protozoan parasites (11, 31). In the case of bacterial infection, type I IFNs seem to have opposing effects depending on the type of bacteria (31). Although exogenous type I IFNs show protective actions in response to infection with *Salmonella typhimurium* or *Shigella flexneri*, the protective effects of endogenous type I IFNs remain unclear (32, 33). In contrast, endogenous type I IFNs reduce resistance to *Listeria monocytogenes* infection (34–36). During infection with the protozoan parasite *Leishmania major*, these exogenous IFNs presumably have a protective effect through the induction of inducible NO synthase, although the involvement of endogenous type I IFNs in antileishmanial immunity is less clear (37, 38). Following infection with *T. cruzi*, administration of exogenous $\alpha\beta$ IFN was reported to reduce the number of serum parasites (10). However, a subsequent study showed that IFNAR1$^{-/-}$ mice were not susceptible to the infection, indicating that endogenous $\alpha\beta$ IFN do not contribute to the host defense against *T. cruzi* (39). Thus, the possible roles of type I IFNs in antitrypanosomal immune responses remain controversial. In the present study, we have clearly established that IFN-β produced by DCs and macrophages contributes to host defense against *T. cruzi*. Thus, endogenous type I IFNs produced during *T. cruzi* infection are responsible for antitrypanosomal immune responses, although the MyD88-dependent production of proinflammatory cytokines overshadows the effects of type I IFNs in normal mice. In the future, it will be interesting to investigate whether this mechanism also applies to immune responses to other protozoan parasites, such as *L. major* and *Toxoplasma gondii*.

We further analyzed the mechanisms by which IFN-β exerts antitrypanosomal responses. The p47 GTPase family members control innate immune responses to intracellular pathogens, including protozoan parasites (27, 28). Expression of p47 GTPases, such as LRG47 and IRG47, and of IGTP is induced through the activation of TLR and IFN signaling pathways during infection with intracellular pathogens. Mice lacking LRG47, IRG47, or IGTP have been shown to become sensitive to infection with *L. major* and *T. gondii*, indicating the possible involvement of these GTPases in *T. cruzi* infection (27, 40, 41). Indeed, LRG47-deficient mice have recently been shown to be sensitive to *T. cruzi* infection (42). We found that induction of IRG47 was impaired in *T. cruzi*-infected cells from MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ mice. Knockdown of IRG47 in MyD88$^{-/-}$ macrophages led to increased intracellular parasites. Thus, TLR-dependent expression of IFN-β probably mediates antitrypanosomal responses through the induction of IRG47.

Recently, MyD88$^{-/-}$TRIF$^{-/-}$ macrophages have been shown to produce IFN-β when infected with intracellular pathogens that escape into the cytosol, such as *L. monocytogenes* and *Legionella pneumophila* (43). In contrast, *T. cruzi*-induced IFN-β production was not observed in MyD88$^{-/-}$TRIF$^{-/-}$ macrophages, although this parasite also invades the cytosol (44). In the case of the cytosolic escape of *Listeria* or *Legionella*, dsDNA from the bacteria is responsible for the induction of IFN-β (43, 45). In contrast to these prokaryotic bacteria, *T. cruzi* is a eukaryote. Therefore, it seems less likely that trypanosomal DNA within the nucleus is exposed to the host cell cytosol, which may lead to the observed absence of TLR-independent induction of IFN-β. Thus, recognition of *T. cruzi* invasion is mainly dependent on TLR systems, possibly at the plasma membrane or in the phagolysosome. However, even in MyD88$^{-/-}$TRIF$^{-/-}$ macrophages, the gene encoding LRG47 was induced after *T. cruzi* infection, indicating the presence of TLR-independent mechanisms for gene expression. The mechanisms for the TLR-independent induction of this p47 GTPase are currently under investigation.

To date, TLR2, TLR4, and TLR9 have been implicated in the recognition of *T. cruzi*-derived components (6, 14–16). TLR2 recognizes GPI-anchored mucin-like proteins and the *T. cruzi*-released protein Tc52 (6, 46, 47), whereas TLR4 is responsible for the recognition of glycoinositolphospholipids (15). TLR9 is also involved in the recognition of the CpG motif present in *T. cruzi* DNA (14). Among these *T. cruzi*-derived components, glycoinositolphospholipids can activate the TRIF-dependent pathway to induce IFN-β via TLR4. It is also possible that currently unknown components are recognized by TLR4 or TLR3, both of which use the TRIF-dependent pathway. Identification of such components responsible for the induction of IFN-β would provide important insights toward understanding innate immune responses to *T. cruzi* infection.

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Disclosures

The authors have no financial conflict of interest.

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nature immunology

Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling

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The Ubc13 E2 ubiquitin-conjugating enzyme is key in the process of 'tagging' target proteins with lysine 63–linked polyubiquitin chains, which are essential for the transmission of immune receptor signals culminating in activation of the transcription factor NF-κB. Here we demonstrate that conditional ablation of Ubc13 resulted in defective B cell development and in impaired B cell and macrophage activation. In response to all tested stimuli except tumor necrosis factor, Ubc13-deficient cells showed almost normal NF-κB activation but considerably impaired activation of mitogen-activated protein kinase. Ubc13-induced activation of mitogen-activated protein kinase required, at least in part, ubiquitination of the adaptor protein IKKγ. These results show that Ubc13 is key in the mammalian immune response.

Stimulation of Toll-like receptors (TLRs), interleukin 1 receptor (IL-1R), antigen receptors, CD40 and tumor necrosis factor receptor (TNFR) results in activation of mitogen-activated protein (MAP) kinases and of the transcription factor NF-κB. Such signals induce immune cell proliferation and survival and cytokine production[1]. In unstimulated cells, IκB proteins sequester NF-κB in the cytoplasm. Immune stimuli result in phosphorylation and ubiquitin- and proteasome-dependent degradation of IκB, thereby permitting translocation of NF-κB to the nucleus[2]. MAP kinases such as c-Jun N-terminal kinase (Jnk) and p38 are rapidly phosphorylated and activated by corresponding 'upstream' MAP kinase kinases, which are activated by MAP kinase kinase kinases. More than ten MAP kinase kinase kinases have been identified[3].

TLRs and IL-1R share 'downstream' signaling molecules, including MyD88, IL-1R-associated kinases (IRAKs) and TNFR-associated factor 6 (TRAF6)[4]. Genetic and biochemical studies suggest that TRAF6, the most distal of these shared signaling proteins, is pivotal in the TLR, IL-1R and CD40 signaling pathways[5]. Moreover, another TRAF family member, TRAF2, is required for TNFR signaling. These observations indicate the convergent function of the TRAF family members in innate immune signaling pathways[6]. Stimulation of B cell receptors (BCRs) and T cell receptors (TCRs) also activates NF-κB and MAP kinases[7]. Adaptor proteins such as Bcl-10, CARMA1 (also called CARD11 or Bimp3) and MALT1 (also called paracaspase) are required for BCR- and TCR-induced NF-κB and MAP kinase activation[8–14]. *In vitro* studies suggest that TRAF2 and TRAF6 are also involved in antigen receptor signaling[15].

TRAF2 and TRAF6 contain N-terminal RING finger domains that have E3 ubiquitin ligase activity[16]. Stimulus-dependent ubiquitination of TRAF6 activates the MAP kinase kinase kinase TGF-β-activated kinase (TAK1), which is critical in the activation of NF-κB and MAP kinases[17–19]. Moreover, TRAF6- and TRAF2-dependent ubiquitination of the adaptor protein IKKγ (also called NEMO) is involved in antigen receptor–induced NF-κB activation[15]. Polyubiquitin chains appended to TRAF and IKKγ are formed through linkages at lysine 63 (K63) of ubiquitin[20].

In contrast to lysine 48 (K48)–linked polyubiquitin chains, which induce proteasome-dependent degradation of the target proteins to which they are appended, K63-linked polyubiquitin chains have been linked to biological processes such as the stress response and DNA repair, rather than protein destruction[21]. K63-linked polyubiquitin chains are reportedly generated by the E2 ubiquitin-conjugating enzyme Ubc13 (ref. 22). The gene encoding Ubc13 was originally identified as being responsible for defective neural development in a drosophila mutant called *bendless*[23]. Subsequently, the synthesis of TRAF2- and TRAF6-dependent K63-linked polyubiquitin chains has been shown to be catalyzed by Ubc13 and Uev1A[16]. RNA silencing of the gene encoding Ubc13 results in defective NF-κB activation in HEK293 cells and insect cells[15,24–27], suggesting that the main function of Ubc13 is in NF-κB activation. In contrast, expression of dominant negative Ubc13 marginally affects TNF-induced NF-κB activation[28],

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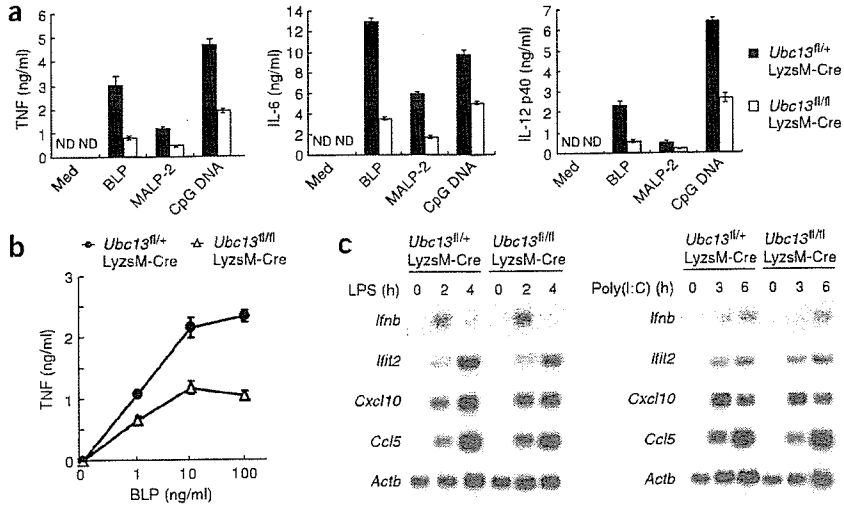

Figure 1 Defective proinflammatory cytokine production in Ubc13-deficient bone marrow macrophages. (**a**) ELISA of IL-6, TNF and IL-12p40 in culture supernatants of bone marrow macrophages (mouse genotypes, key) cultured for 24 h with 100 ng/ml of BLP, 30 ng/ml of MALP-2 or 1 μM CpG DNA in the presence of 30 ng/ml of interferon-γ. Med, medium only; ND, not detected. (**b**) ELISA of TNF in culture supernatants of bone marrow macrophages (mouse genotypes, key) cultured for 24 h with BLP (concentration, horizontal axis) in the presence of 30 ng/ml of interferon-γ. Data (**a**,**b**) represent mean ± s.d. of triplicate samples and are representative of three independent experiments. (**c**) RNA blot analysis of total RNA (10 μg) extracted from bone marrow macrophages (mouse genotypes, above blots) stimulated with 100 ng/ml of LPS (left) or 50 μg/ml of poly(I:C) (right). *Ifnb*, *Ifit2*, *Cxcl10*, *Ccl5* and *Actb* encode interferon-β, ISG54, IP-10, RANTES and β-actin, respectively. Data are representative of two independent experiments.

and the RING finger domain of TRAF6, which is essential for ligation of K63-linked polyubiquitin chains to target proteins, is dispensable for the IL-1R- and TLR-mediated NF-κB activation[29]. Those results suggest that Ubc13 has a minor function in NF-κB activation. Thus, whether Ubc13 is essential for immune signaling and immune responses *in vivo* is not known.

Here we have generated mice conditionally deficient in Ubc13. We demonstrate that Ubc13 was essential for TLR-induced proinflammatory cytokine production in bone marrow–derived macrophages. In addition, Ubc13 was required for TLR-, CD40- and BCR-induced B cell activation. B cell–specific deletion of Ubc13 resulted in defective development of marginal zone B cells and B-1 cells and in impaired humoral immune responses. Ubc13-deficient cells had almost normal NF-κB activation and normal TAK1 phosphorylation. In contrast, Ubc13-deficient cells had substantially impaired MAP kinase activation in response to all stimuli tested, except for TNF. Ubc13-induced MAP kinase activation was mediated partially through ubiquitination of IKKγ, which was abolished in Ubc13-deficient cells. Our results demonstrate the physiological importance of Ubc13 in the induction of mammalian immune responses.

RESULTS

Conditional ablation of Ubc13

To assess the function of Ubc13 in adult mice, we generated mice in which Ubc13 could be conditionally ablated (**Supplementary Fig. 1** online). The gene encoding Ubc13 (called '*Ubc13*' here) consists of four exons. We constructed a targeting vector to insert *lox*P sites flanking exons 2, 3 and 4 of *Ubc13* and to insert a *lox*P-flanked neomycin-resistance gene into intron 1 of *Ubc13*. To generate conventional Ubc13-deficient ($Ubc13^{-/-}$) mice, we constructed another targeting vector lacking the flanked exons (data not shown). In both cases, we microinjected two correctly targeted embryonic stem cell clones into C57BL/6 blastocysts to generate chimeric mice. We crossed male chimeric with female C57BL/6 mice and monitored transmission of the mutated allele by Southern blot analysis (**Supplementary Fig. 1** and data not shown). Although $Ubc13^{+/-}$ mice were phenotypically normal and fertile, we failed to obtain $Ubc13^{-/-}$ offspring by intercrossing $Ubc13^{+/-}$ mice (**Supplementary Table 1** online). To determine the time of death *in utero*, we genotyped embryos from $Ubc13^{+/-}$ intercrosses at embryonic day 13.5 or 9.5. We detected no $Ubc13^{-/-}$ embryos, indicating that Ubc13 deficiency results in early embryonic death. In contrast, mice homozygous for *lox*P-flanked *Ubc13* alleles ($Ubc13^{fl/fl}$ mice) were born at the expected mendelian ratios and had no obvious abnormalities (data not shown).

Ubc13 in macrophage activation

Because Ubc13 has been linked to the activation of TRAF6, a crucial component of TLR signaling pathways[16,30], we assessed the function of Ubc13 in the TLR responses in bone marrow–derived macrophages. To disrupt *Ubc13* specifically in macrophages, were crossed $Ubc13^{fl/fl}$ mice with mice in which cDNA encoding Cre recombinase is inserted into the gene encoding lysozyme M, which is specifically expressed in the myeloid linage such as macrophages and granulocytes (LyzsM-Cre mice). Southern blot analysis showed that in bone marrow macrophages from the resultant '$Ubc13^{fl/fl}$LyzsM-Cre mice', Cre-mediated deletion produced a new 1.1-kb band corresponding to the mutated *Ubc13* allele, and immunoblot analysis showed that $Ubc13^{fl/fl}$LyzsM-Cre bone marrow macrophages had much less Ubc13 protein than did control cells (**Supplementary Fig. 1**).

Bone marrow macrophages produce proinflammatory cytokines in response to a variety of TLR ligands in a MyD88-dependent way[4]. Thus, we assessed cytokine production by $Ubc13^{fl/fl}$LyzsM-Cre bone marrow macrophages stimulated with TLR ligands such as BLP, MALP-2 and CpG DNA. Bone marrow macrophages from $Ubc13^{fl/fl}$LyzsM-Cre mice produced less TNF, IL-6 and IL-12p40 than did those from control mice (**Fig. 1a**), and the response to BLP was dose dependent (**Fig. 1b**). These results indicate that Ubc13 is important in TLR-induced cytokine production in bone marrow macrophages.

TLR signaling can be MyD88 dependent or MyD88 independent. MyD88-independent TLR3 and TLR4 signaling results in the induction of type I interferon and interferon-inducible genes[4]. To assess the function of Ubc13 in MyD88-independent immune responses, we analyzed expression of the gene encoding interferon-β and of interferon-inducible genes, including *Ifit2*, *Cxcl10* and *Ccl5*, after treatment of control or $Ubc13^{fl/fl}$LyzsM-Cre bone marrow macrophages with lipopolysaccharide (LPS) or poly(I:C). Control and $Ubc13^{fl/fl}$LyzsM-Cre bone marrow macrophages contained similar amounts of transcripts encoding interferon and of interferon-inducible gene transcripts after LPS or poly(I:C) stimulation, indicating that

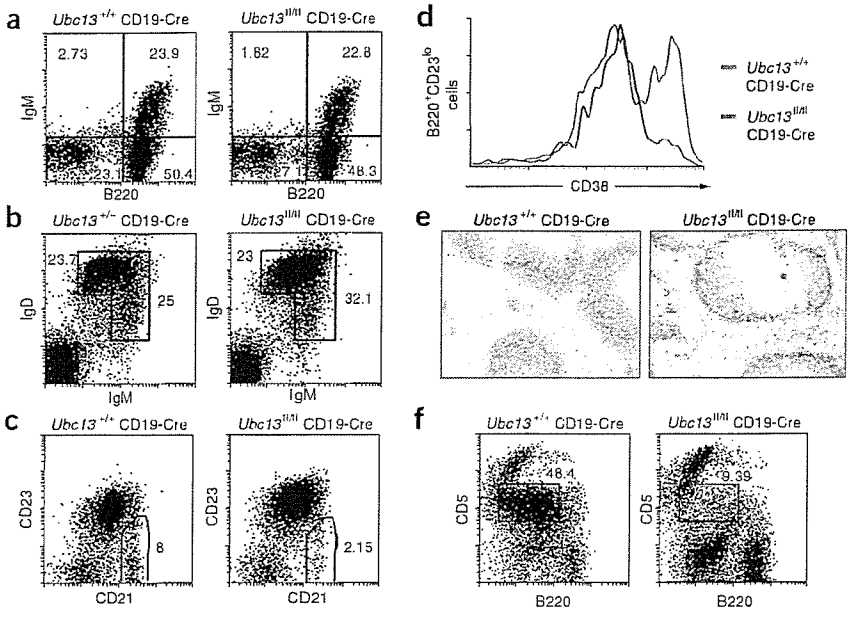

Figure 2 Impaired B cell development in *Ubc13*$^{fl/fl}$Cd19-Cre mice. (**a–d**) Flow cytometry of IgM and B220 expression by B cell precursors in the bone marrow (**a**) and of IgM and IgD expression (**b**), CD21 and CD23 expression by B220$^+$ populations (**c**) and CD38 expression by B220$^+$CD23^{lo} populations (**d**) of B cells in the spleens of 6- to 10-week-old mice. (**e**) Frozen splenic sections stained with rat monoclonal antibody to mouse metallophilic macrophages (red) and with anti-B220 to visualize B cells (blue). Original magnification, ×200. (**f**) Flow cytometry of B220 and CD5 expression by CD5$^+$ B cells in the peritonea of 6- to 10-week-old mice. Numbers in dot plots indicate percentages of cells in each quadrant (**a**); of mature (**b**, left) and immature (**b**, right) splenic B cells; of marginal zone B cells (**c**); and of B1 cells (**f**). Data are representative of three independent experiments.

Ubc13 is dispensable for the TLR-mediated MyD88-independent immune responses in bone marrow macrophages (**Fig. 1c**).

Ubc13 in B cell development and function

Mice lacking molecules involved in BCR signaling show defective B cell development. Specifically, mice lacking the Bcl-10 or MALT1 adaptor proteins show defective development of marginal zone B cells and B-1 B cells[9,13,31]. The adaptor protein CARMA1 is also essential for the generation of B-1 B cells[10–12,14]. To determine whether Ubc13 deficiency affects the B cell development, we generated mice lacking Ubc13 specifically in the B cell lineage. We crossed *Ubc13*$^{fl/fl}$ mice with mice expressing a Cre transgene under control of the *Cd19* promoter (Cd19-Cre mice). Southern blot and immunoblot analysis showed almost complete Cre-mediated deletion of the *Ubc13 lox*P-flanked alleles and the protein in splenic B220$^+$ cells from these '*Ubc13*$^{fl/fl}$ Cd19-Cre mice' (**Supplementary Fig. 1**).

To determine whether the Ubc13 disruption affected B cell development, we examined the bone marrow of control and *Ubc13*$^{fl/fl}$Cd19-Cre mice. Control and *Ubc13*$^{fl/fl}$Cd19-Cre mice had no differences in bone marrow cellularity, and B cell precursor populations in control and *Ubc13*$^{fl/fl}$Cd19-Cre mice had similar expression of surface B220 and immunoglobulin M (IgM; **Fig. 2a**). Moreover, splenocytes from *Ubc13*$^{fl/fl}$Cd19-Cre mice had a pattern of surface expression of CD3 and B220 similar to that of control mice (**Supplementary Fig. 2** online). Whereas the expression of IgM and IgD was similar on the surfaces of splenocytes from control and *Ubc13*$^{fl/fl}$Cd19-Cre mice (**Fig. 2b**), *Ubc13*$^{fl/fl}$Cd19-Cre mice had a much lower frequency of B220$^+$CD21^{hi}CD23^{lo} marginal zone B cells (**Fig. 2c**). Detection of marginal zone B cells with another set of surface antigens, B220, CD38 and CD21, produced similar results (**Fig. 2d**). Immunohistochemical staining confirmed that the width of marginal zone B cell area was smaller in spleens from *Ubc13*$^{fl/fl}$Cd19-Cre mice (**Fig. 2e**). The CD5$^+$ peritoneal B-1 cell population was also much lower in *Ubc13*$^{fl/fl}$Cd19-Cre mice (**Fig. 2f**). These results suggest that Ubc13 is essential for the development of marginal zone B cells and peritoneal CD5$^+$ B-1 cells.

To test whether Ubc13 is involved in TLR responses in B cells, we analyzed the proliferation of control and *Ubc13*$^{fl/fl}$Cd19-Cre B cells stimulated with LPS or CpG DNA. Control B cells proliferated in a dose-dependent way in response to both LPS and CpG DNA stimulation (**Fig. 3a**). In contrast, proliferation of *Ubc13*$^{fl/fl}$Cd19-Cre B cells in response to these stimuli was much lower. In addition, CpG DNA–induced IL-6 production in *Ubc13*$^{fl/fl}$Cd19-Cre B cells was severely impaired (**Fig. 3b**). Compared with control B cells, *Ubc13*$^{fl/fl}$Cd19-Cre B cells also showed defective proliferation in response to stimulation with antibody to IgM (anti-IgM) or anti-CD40 (**Fig. 3a**). We next assessed whether these defects in TLR-, BCR- and CD40-mediated proliferation were accompanied by impaired cell cycle progression in *Ubc13*$^{fl/fl}$Cd19-Cre B cells. Compared with control B cells, which entered S phase after stimulation with LPS, CpG DNA, anti-IgM or anti-CD40, fewer *Ubc13*$^{fl/fl}$Cd19-Cre B cells entered S phase after stimulation (**Fig. 3c**). Stimulation with LPS, CpG DNA or anti-CD40 can prevent B cell apoptosis that normally results from *ex vivo* culture of B cells without mitogens[19]. A greater proportion of *Ubc13*$^{fl/fl}$Cd19-Cre B cells than control B cells underwent apoptosis *ex vivo*, even after stimulation with LPS, CpG DNA or anti-CD40 (**Fig. 3d**). These results collectively suggest that Ubc13 is critical for TLR-, BCR-, and CD40-mediated B cell activation, proliferation and survival.

To investigate whether the defective activation and development of *Ubc13*$^{fl/fl}$Cd19-Cre B cells affected on the immune responses *in vivo*, we compared immunoglobulin concentrations in sera of control and *Ubc13*$^{fl/fl}$Cd19-Cre mice (**Fig. 3e**). All immunoglobulin isotypes tested except IgG2a and IgG2b were significantly lower in *Ubc13*$^{fl/fl}$ Cd19-Cre mice than in control mice. After immunization with the T cell–independent polyvalent antigen trinitrophenol-Ficoll or the T cell–dependent antigen nitrophenol–chicken γ-globulin, *Ubc13*$^{fl/fl}$ Cd19-Cre mice had significantly less serum trinitrophenol-specific IgM and IgG3 (**Fig. 3f**). Although nitrophenol-specific IgM titers were similar, nitrophenol-specific IgG1 titers were lower in *Ubc13*$^{fl/fl}$Cd19-Cre mice (**Fig. 3g**). Thus, Ubc13 is required for appropriate humoral immune responses *in vivo*.

Ubc13 in NF-κB and MAPK activation

TLR, BCR, CD40, IL-1R and TNFR signals culminate in NF-κB activation[32]. Although *in vitro* studies indicate that Ubc13 is involved in NF-κB activation in HEK293 cells[33], whether Ubc13 deficiency affects NF-κB activation mediated by TLR, BCR, CD40, TNFR or IL-1R in other cell types in physiological conditions is not known.

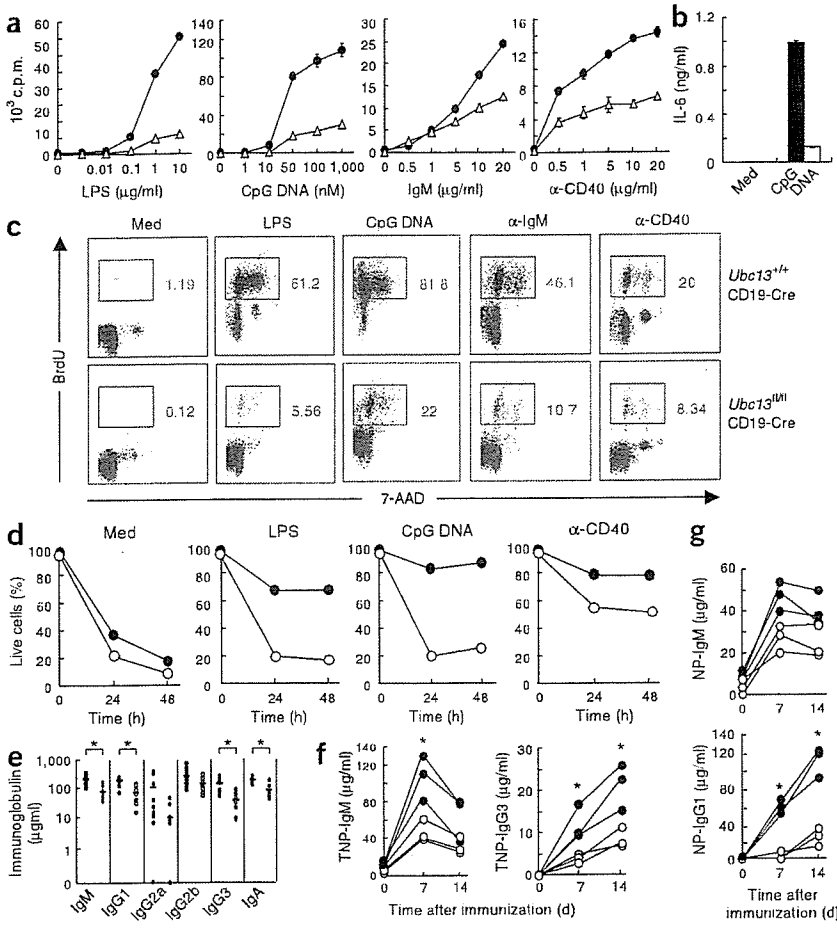

Figure 3 Ubc13 is required for B cell activation and *in vivo* immune responses. **(a)** Proliferation of splenic B220$^+$ B cells cultured for 48 h with various stimuli (horizontal axes). α-CD40, anti-CD40. Data are representative of three independent experiments. **(b)** ELISA of IL-6 in supernatants of splenic B220$^+$ B cells stimulated for 48 h with 1 μM CpG DNA. ND, not detected. Data represent mean ± s.d. of triplicate samples and are representative of two independent experiments. **(c)** Cell cycle profiles of B cells stimulated with 10 μg/ml of LPS, 1 μM CpG DNA, 10 μg/ml of anti-IgM or 10 μg/ml of anti-CD40. Cells labeled with 5-bromodeoxyuridine (BrdU) and 7-amino-actinomycin D (7-AAD) were analyzed by flow cytometry 24 h after stimulation. Numbers beside boxed areas indicate percentages of cells in S phase. Data are representative of two independent experiments. **(d)** Viability of B cells stimulated with 10 μg/ml of LPS, 1 μM CpG DNA or 10 μg/ml of anti-CD40, as assessed by annexin V staining (time, horizontal axes). Data are representative of three independent experiments. **(e)** ELISA of immunoglobulin isotypes in the sera of unimmunized 8-week-old mice ($n = 10$ mice of each genotype). Each symbol represents one mouse. *, $P < 0.05$ (Student's *t*-test). **(f)** Production of trinitrophenol-specific IgM (TNP-IgM) and IgG3 (TNP-IgG3) at 7 and 14 d after immunization with trinitrophenol-Ficoll. **(g)** Production of nitrophenol-specific IgM (NP-IgM) and IgG1 (NP-IgG1) at 7 and 14 d after immunization with nitrophenol–chicken γ-globulin. Results in **f,g** represent three of five mice per genotype; *, $P < 0.05$ (Student's *t*-test). Filled symbols or bars, *Ubc13*$^{+/+}$Cd19-Cre; open symbols or bars, *Ubc13*$^{fl/fl}$Cd19-Cre.

LPS, CpG DNA, anti-IgM and anti-CD40 resulted in slightly defective IκBα degradation but normal NF-κB nuclear translocation in *Ubc13*$^{fl/fl}$Cd19-Cre B cells (**Fig. 4a,b**). Nuclear NF-κB complexes contained similar NF-κB subunits in wild-type and *Ubc13*$^{fl/fl}$Cd19-Cre B cells (**Supplementary Fig. 3** online). In addition, BLP- and CpG DNA–mediated IκBα degradation NF-κB nuclear translocation was indistinguishable in control versus *Ubc13*$^{fl/fl}$LyzsM-Cre bone marrow macrophages (**Fig. 4c,d**). To determine whether Ubc13 is involved in TNFR- and IL-1R-mediated signal transduction, we generated mouse embryonic fibroblasts (MEFs) from control and *Ubc13*$^{fl/fl}$ embryos. Retroviral transduction of Cre into *Ubc13*$^{fl/fl}$ MEFs induced efficient deletion of Ubc13 protein (**Supplementary Fig. 1**). *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre had normal TNF- and IL-1β-induced IκBα degradation and NF-κB nuclear translocation (**Fig. 4e,f**). To further investigate whether Ubc13 deficiency is dispensable for NF-κB activation, we transfected an NF-κB-dependent luciferase reporter construct into control or *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre. TNF or IL-1β stimulation induced similar luciferase activity in control and *Ubc13*$^{fl/fl}$ MEFs (**Fig. 4g**). Moreover, overexpression of Bcl-10 or CARMA1 resulted in similar NF-κB activation in control and *Ubc13*$^{fl/fl}$ MEFs (**Fig. 4h**). In addition, IL-1β-induced IκBα recovery at later time points was similar for control and *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre (**Fig. 4i**). To analyze the function of Ubc13 in alternative pathways of NF-κB activation, we assessed stimulus-dependent processing of the NF-κBp100 subunit. Control and *Ubc13*$^{fl/fl}$Cd19-Cre B cells had similar patterns of processing of NF-κBp100 to NF-κBp52 after stimulation with anti-CD40 or B cell–activating factor of the TNF family (**Supplementary Fig. 3**), indicating that Ubc13 is not involved in the alternative pathway of NF-κB activation. These data collectively suggest that although Ubc13 deficiency slightly affected IκBα degradation in some cell types, Ubc13 seems to be mostly dispensable for NF-κB activation.

Next we investigated activation of the Jnk, p38 and Erk MAP kinases in B cells, bone marrow macrophages and MEFs. Stimulation of control B cells with anti-IgM resulted in phoshorylation of Jnk, p38 and Erk. In contrast, although Erk activation was normal, anti-IgM-induced phoshorylation of Jnk and p38 was substantially impaired in *Ubc13*$^{fl/fl}$Cd19-Cre B cells. Moreover, *Ubc13*$^{fl/fl}$Cd19-Cre B cells had considerably reduced activation of all three MAP kinases in response to LPS and CpG DNA, and anti-CD40-induced MAP kinase activation was also mildly affected by Ubc13 deficiency (**Fig. 5a**). Although stimulation with BLP or CpG DNA resulted in rapid phosphorylation of MAP kinases in control bone marrow macrophages, MAP kinase activation in *Ubc13*$^{fl/fl}$LyzsM-Cre bone marrow macrophages was impaired (**Fig. 5b**). Furthermore, IL-1β-induced phosphorylation of Jnk, p38 and Erk was much lower in *Ubc13*$^{fl/fl}$ than in control MEFs expressing retroviral Cre (**Fig. 5c**). In contrast, TNF stimulation resulted in similar MAP kinase activation in *Ubc13*$^{fl/fl}$ and control MEFs expressing retroviral Cre (**Fig. 5d**). These results collectively demonstrate that Ubc13 is important in MAP kinase activation induced by TLR, IL-1R, BCR and CD40, but not MAP kinase activation induced by TNFR.

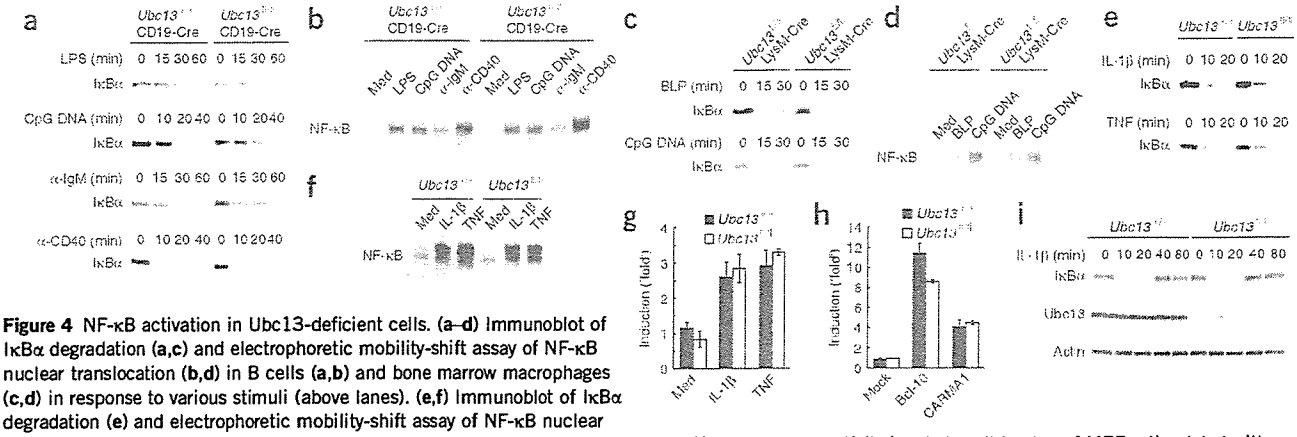

Figure 4 NF-κB activation in Ubc13-deficient cells. (**a–d**) Immunoblot of IκBα degradation (**a,c**) and electrophoretic mobility-shift assay of NF-κB nuclear translocation (**b,d**) in B cells (**a,b**) and bone marrow macrophages (**c,d**) in response to various stimuli (above lanes). (**e,f**) Immunoblot of IκBα degradation (**e**) and electrophoretic mobility-shift assay of NF-κB nuclear translocation (**f**) in MEFs in response to various stimuli (above lanes). (**g**) NF-κB luciferase reporter activity in whole-cell lysates of MEFs stimulated with 10 ng/ml of IL-1β or 10 ng/ml of TNF. (**h**) NF-κB luciferase reporter activity in whole-cell lysates of MEFs transfected with plasmids encoding Bcl-10 or CARMA1. (**i**) Immunoblot of IκBα degradation and recovery in MEFs stimulated with 10 ng/ml of IL-1β (time, above lanes). Data are representative of two (**i**) or three (**a–h**) independent experiments.

These data indicate that Ubc13 is critically involved in MAP kinase activation, yet has only a minor function in NF-κB activation. To elucidate precisely where in the TLR, IL-1R, BCR and CD40 signaling pathways Ubc13 induces MAP kinase activation, we examined the activation and modification of signaling molecules 'upstream' of the IKK complex and MAP kinase kinase proteins. *In vitro* studies indicate that IL-1β stimulates TRAF6 ubiquitination in a Ubc13-dependent way[16]. Therefore, we examined IL-1β-induced polyubiquitination of TRAF6 in control or *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre. IL-1β-induced polyubiquitination of TRAF6 was evident in control and *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre (**Fig. 6a**). As the K63-linked ubiquitination of TRAF6 has been linked to activation of the kinase TAK1 (ref. 17), we next examined IL-1β-mediated TAK1 phosphorylation in control and *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre. These MEFs had similar amounts of phosphorylated TAK1, albeit with delayed kinetics in *Ubc13*$^{fl/fl}$ MEFs compared with control MEFs (**Fig. 6b**). Next we undertook a biochemical approach in which we added recombinant TRAF6 to extracts from unstimulated control cells, which results in phosphorylation of TAK1 and IKK proteins[16,17] (**Fig. 6c**). The addition of TRAF6 to extracts of *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre resulted in slightly impaired TAK1 phosphorylation and normal phosphorylation of IKK proteins. These results suggested that Ubc13 is involved in TAK1 activation but that Ubc13 is not absolutely essential for IL-1β-induced TAK1 activation.

Given that TAK1 is critical in IL-1β-induced activation of NF-κB and MAP kinases[18,19] and that there was considerable activation of TAK1 and NF-κB in *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre, TAK1 activation might contribute to the substantial IL-1β-induced NF-κB

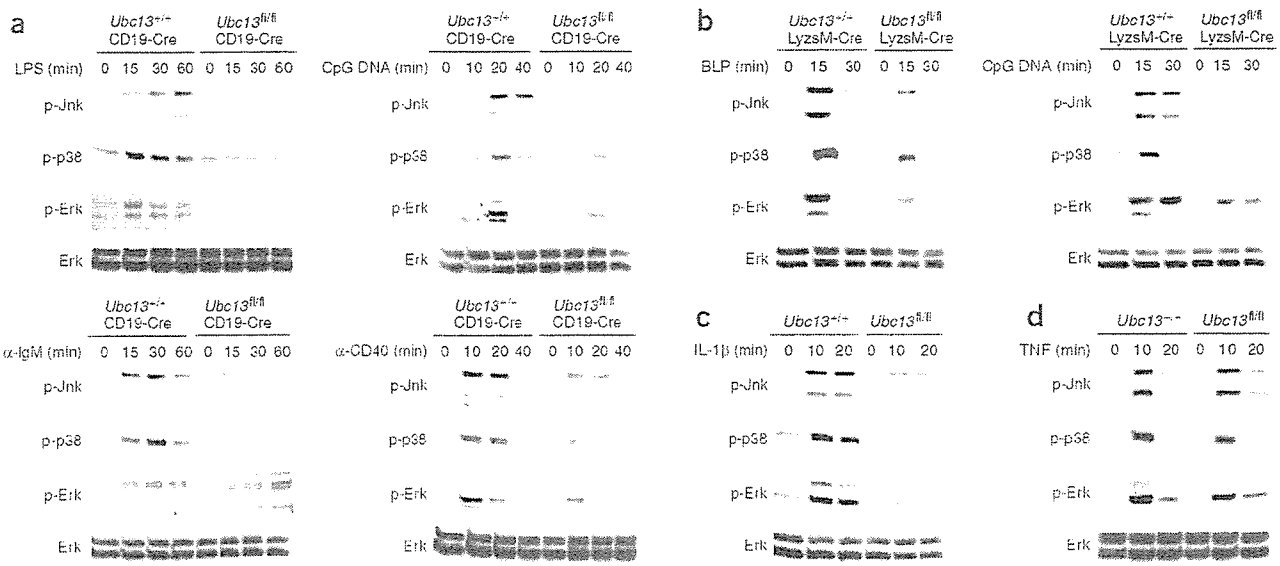

Figure 5 Impaired MAP kinase activation in Ubc13-deficient cells. (**a**) Immunoblot of whole-cell lysates of B cells stimulated with 10 μg/ml of LPS, 1 μM CpG DNA, 10 μg/ml of anti-IgM or 10 μg/ml of anti-CD40. (**b**) Immunoblot of whole-cell lysates of bone marrow macrophages stimulated with 100 ng/ml of BLP or 1 μM CpG DNA. (**c,d**) Immunoblot of whole-cell lysates of MEFs stimulated with 10 ng/ml of IL-1β (**c**) or 10 ng/ml of TNF (**d**). p-, phosphorylated. Data are representative of three independent experiments.

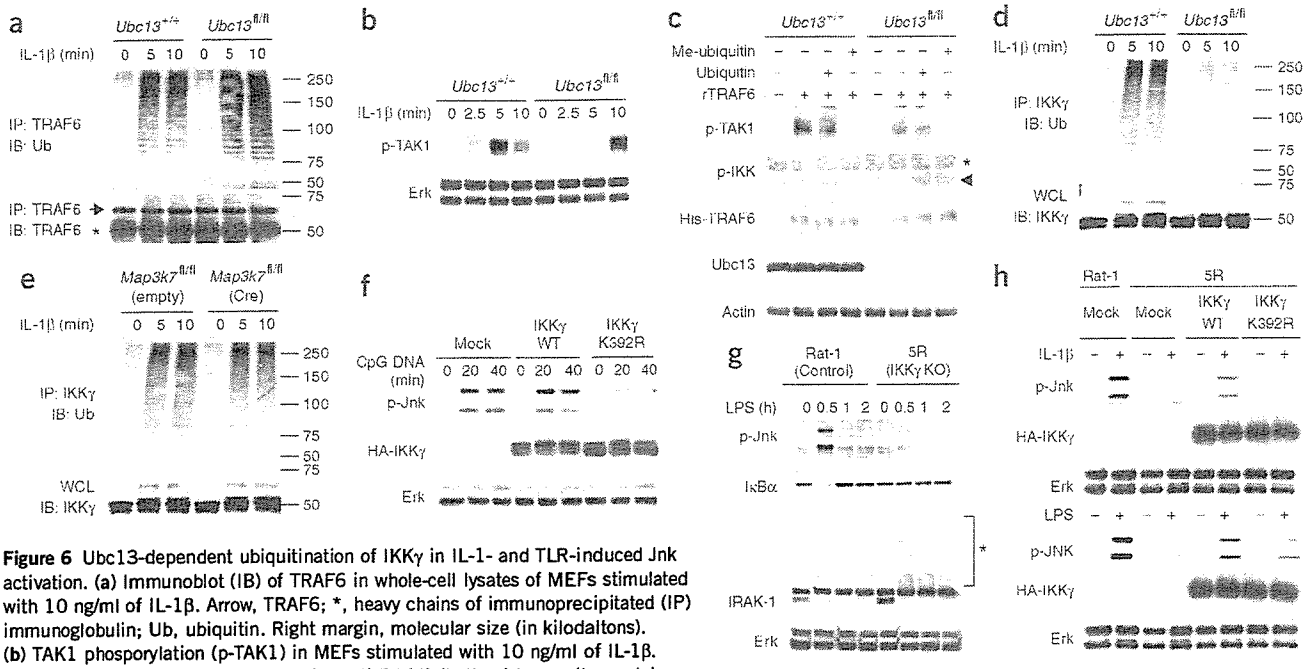

Figure 6 Ubc13-dependent ubiquitination of IKKγ in IL-1- and TLR-induced Jnk activation. **(a)** Immunoblot (IB) of TRAF6 in whole-cell lysates of MEFs stimulated with 10 ng/ml of IL-1β. Arrow, TRAF6; *, heavy chains of immunoprecipitated (IP) immunoglobulin; Ub, ubiquitin. Right margin, molecular size (in kilodaltons). **(b)** TAK1 phosporylation (p-TAK1) in MEFs stimulated with 10 ng/ml of IL-1β. The same lysates were also blotted with anti-Erk1/2 (bottom) to monitor protein expression. **(c)** Immunoblot of cell extracts of MEFs incubated with recombinant TRAF6 (rTRAF6) in the presence of ubiquitin or methylated ubiquitin (Me-ubiquitin). Arrowhead, phosphorylated IKK (p-IKK); *, nonspecific bands; His-, histidine-tagged. **(d)** Immunoblot of IKKγ immunoprecipitates from whole-cell lysates of MEFs stimulated with 10 ng/ml of IL-1β. **(e)** Immunoblot of IKKγ immunoprecipitates from whole-cell lysates of MEFs stimulated with 10 ng/ml of IL-1β. *Map3k7*$^{fl/fl}$, *lox*P-flanked gene encoding TAK1, with (Cre) and without (empty) Cre recombinase. WCL (bottom, **d,e**), immunoblot of whole-cell lysates without immunoprecipitation. **(f)** Immunoblot of whole-cell lysates from 70Z/3 pre–B cell lines stably transfected with wild-type (WT) or K392R IKKγ expression vectors. HA-, hemagglutinin-tagged. **(g)** Immunoblot of whole-cell lysates of Rat-1 or 5R cells stimulated with 10 μg/ml of LPS. *, ubiquitinated IRAK-1. **(h)** Immunoblot of whole-cell lysates of Rat-1 or 5R cells stably expressing wild-type or K392R IKKγ expression vectors, stimulated with 10 ng/ml of IL-1β (top) or 10 μg/ml of LPS (bottom). Data are representative of two (**a,b,d,f,g,h**) or three (**c,e**) independent experiments.

activation in the absence of Ubc13. However, given the impaired IL-1β-induced MAP kinase activation in Ubc13-deficient cells, full activation of TAK1 alone may be insufficient for full activation of MAP kinases. Ubc13 has also been suggested to be involved in TCR-induced ubiquitination of IKKγ[15]. We found that IL-1β also stimulated IKKγ polyubiquitination in control MEFs, but that IL-1β-induced IKKγ polyubiquitination was impaired in *Ubc13*$^{fl/fl}$ MEFs expressing retroviral Cre (**Fig. 6d**). Moreover, IL-1β-induced polyubiquitination of IKKγ was normal in TAK1-deficient MEFs (**Fig. 6e**), suggesting that the Ubc13-dependent ubiquitination of IKKγ is a TAK1-independent signaling event.

These aforementioned results prompted us to examine whether the ubiquitination of IKKγ is involved in TLR- and IL-1R-induced MAP kinase activation. As lysine 392 (K392) of mouse IKKγ is known to be the acceptor lysine residue for Ubc13-dependent polyubiquitination[24], we generated a K392R mouse IKKγ mutant and compared CpG DNA–induced Jnk activation in the mouse pre–B cell line 70Z/3 expressing wild-type or K392R IKKγ constructs. The 70Z/3 cells expressing K392R IKKγ had defective activation of Jnk in response to CpG DNA relative to that of mock-transfected 70Z/3 cells or 70Z/3 cells expressing wild-type IKKγ, suggesting that IKKγ K392R inhibits CpG DNA–mediated Jnk activation in a dominant negative way (**Fig. 6f**). Moreover, activation of Jnk and p38 induced by CpG DNA or by phorbol 12-myristate 13-acetate plus ionomycin was impaired in IKKγ-deficient 1.3E2 cells relative to that in control 70Z/3 cells (**Supplementary Fig. 4** online). We further investigated stimulus-dependent activation of MAP kinases in other cell types. MAP kinase activation in response to LPS or IL-1β was reduced considerably in the rat IKKγ-deficient fibroblast cell line 5R as well as in IKKγ-deficient MEFs (**Fig. 6g** and **Supplementary Fig. 4**), indicating that IKKγ is essential for full activation of MAP kinases in response to multiple immune stimuli. To clarify whether ubiquitination of IKKγ is necessary for full MAP kinase activation, we retrovirally expressed wild-type or K392R IKKγ in the IKKγ-deficient 5R fibroblasts and analyzed IL-1β- and LPS-induced Jnk activation. Ectopic expression of wild-type IKKγ induced considerable Jnk activation (**Fig. 6h**). In contrast, K392R IKKγ permitted much weaker Jnk activation. These results indicate that Ubc13-dependent IKKγ ubiquitination may have a partial function in IL-1R- and TLR-induced MAP kinase activation.

DISCUSSION

Here we used a conditional ablation strategy to analyze the physiological function of Ubc13. Ubc13-deficient cells showed almost normal NF-κB activation and severely impaired MAP kinase activation in response to a variety of stimuli, except for TNF. *Ubc13* disruption in bone marrow macrophages resulted in impaired TLR-induced proinflammatory cytokine production and MAP kinase activation. Macrophages treated with MAP kinase inhibitors show defective cytokine production[34,35] and cells lacking MAP kinase phosphatase 5, which show augmented stimulus-dependent Jnk activation, conversely produce increased amounts of cytokines[36], suggesting that MAP kinase activation promotes TLR-induced cytokine production. Failure to activate MAP kinases after TLR stimulation

may have contributed to the reduced cytokine production in Ubc13-deficient bone marrow macrophages. However, there was normal TLR-mediated MyD88-independent expression of type I interferon and interferon-inducible genes in Ubc13-deficient cells, suggesting that Ubc13 is dispensable for TLR-mediated MyD88-independent immune responses.

Ubc13-deficient B cells showed impaired BCR-, CD40- and TLR-mediated activation. In terms of B cell development, mice lacking components of the BCR signaling pathway have phenotypes similar to that of *Ubc13*$^{fl/fl}$Cd19-Cre mice. For example, development of marginal zone B cells and B-1 cells is compromised in *Bcl10*$^{-/-}$ mice and *Malt1*$^{-/-}$ mice[9,13,31]. Although investigation of marginal zone B cells in CARMA1-deficient (*Card11*$^{-/-}$) mice has not been reported, these mice have substantially impaired development of B-1 cells[10–12,14]. Moreover B-1 cell populations are considerably reduced in the peritoneal cavities of mice lacking TAK1, which also participates in BCR signaling[19,37]. Thus, these similarities indicate that Ubc13 may be involved in BCR signaling pathways that are essential for the development of certain B cell lineages. Contrary to published results showing that Ubc13 is involved in TNFR- and TRAF2-dependent activation of NF-κB or MAP kinases[16,28,38], Ubc13-deficient MEFs showed normal TNF-induced activation of NF-κB and MAP kinases, indicating that Ubc13 might have a minor function in TNFR-mediated activation in this cell type.

In response to IL-1β and TLR ligands, Ubc13-deficient cells showed almost normal NF-κB activation and considerable impairment in MAP kinase activation. In addition, there was unexpected IL-1β-induced polyubiquitination of TRAF6 in Ubc13-deficient MEFs. *In vitro* ubiquitination assays in which only Ubc13 and Uev1A E2 ubiquitin ligases are present have shown that TRAF2 and TRAF6 are involved in the generation of K63-linked but not K48-linked polyubiquitin chains[15,16]. However, given that overexpression of TRAF2 *in vivo* generates K63- as well as K48-linked ubiquitination[28], IL-1β-induced Ubc13-independent K48-linked but not Ubc13-dependent K63-linked ubiquitination of TRAF6 might be detected in Ubc13-deficient MEFs. Alternatively, another E2 conjugating enzyme, UbcH7, has been shown to facilitate TRAF6-related K63-linked polyubiquitination[39]. UbcH7 might compensate for the lack of Ubc13 and stimulate IL-1β-induced K63-linked ubiquitination of TRAF6.

Ubc13-dependent ubiquitination is involved in TAK1 activation[17]. Here, Ubc13-deficient MEFs showed almost normal TAK1 phosphorylation, albeit with substantially delayed kinetics, suggesting that Ubc13 is not absolutely required for full activation of TAK1. Analysis of TAK1-deficient cells has demonstrated that TAK1 is essential in TLR- and IL-1R-induced activation of NF-κB and MAP kinases[18,19]. Therefore, we propose that although full TAK1 activation alone may be sufficient to induce considerable NF-κB activation in response to IL-1β, supplemental signaling events 'downstream' of or independent of TAK1 might also be required for the efficient activation of MAP kinases.

Several *in vitro* experiments have demonstrated that IKKγ is ubiquitinated in a Ubc13-dependent way[15,24,27]. In our study here, IL-1β-mediated ubiquitination of IKKγ was considerably impaired in Ubc13-deficient MEFs but was normal in TAK1-deficient cells, suggesting possible involvement of IKKγ ubiquitination in MAP kinase activation. Overexpression of the ubiquitination-deficient K392R IKKγ mutant reduced Jnk activation in 70Z/3 cells. In addition, the derivative IKKγ-deficient cell line 1.3E2 showed defective Jnk and p38 activation in response to CpG DNA (or phorbol 12-myristate 13-acetate plus ionomycin). Indeed, characterization of IKKγ-deficient 1.3E2 cells has demonstrated 'normal' MAP kinase activation in response to stimulation with phorbol 12-myristate 13-acetate plus ionomycin[40]. In contrast, another study has shown that although 1.3E2 cells have impaired Jnk activation in response to the same stimuli tested in that previous study[40], responsiveness is restored by ectopic expression of protein kinase C-θ (PKC-θ), PKC-δ or IKKγ; that study concluded that 1.3E2 cells lack PKC-θ and PKC-δ in addition to PKC-θ and PKC-δ[41]. However, as we reproducibly found impairment in the activation of Jnk and p38 in several IKKγ-deficient cell lines, we believe that IKKγ might be involved in the stimulus-dependent activation of the MAP kinases as well as NF-κB in these cell types. Furthermore, re-expression of the K392R IKKγ mutant, to which K63-linked polyubiquitin chains cannot be appended, in IKKγ-deficient 5R cells conferred much weaker Jnk activation in response to IL-1β and LPS than did wild-type IKKγ, suggesting that Ubc13-dependent IKKγ ubiquitination may be essential at least in part for Jnk activation. The IKKγ-interacting protein Act1 (also called CIKS), whose overexpression potentiates Jnk and NF-κB activation, might be involved in IKKγ-related Jnk activation[42,43]. Studies using Act1-deficient cells may allow examination of that possibility[44].

Although IKKγ deficiency resulted in defective activation of MAP kinases in our study, IKKγ-deficient cell lines may have mutated in the interim between previously published studies and our studies here, resulting in loss of the original phenotype of 'normal' MAP kinase activation reported before[40]. Further analysis using 'knock-in' mice homozygous for the mutation producing the IKKγ K392R substitution will provide physiological conditions in which to examine whether Ubc13-dependent ubiqituitination of IKKγ is involved in the activation of MAP kinases (as well as NF-κB) in response to various stimuli in other cell types and immune responses.

As for the involvement of Ubc13 in NF-κB activation, given that mice lacking either IKKβ or IKKγ, both of which are essential for BCR-induced NF-κB activation, also show defects in the development of marginal zone B cells and B-1 cells[45,46], Ubc13 might be critical to IKKβ-dependent NF-κB activation in a cell type–specific way. Moreover, we cannot exclude the possibility that conditional deletion of Ubc13 may have been incomplete in some cell types or that another E2 family member (such as Ubc5, which is involved in IκBα phosphorylation[47] and K63-linked polyubiquitin chain synthesis[48]) compensated for the loss of Ubc13 in some cell types. Further studies are needed to comprehensively evaluate the function of Ubc13 in NF-κB activation using other cell types such as T cells, hepatocytes and keratinocytes.

In summary, here we have provided genetic evidence that Ubc13 is involved in TLR-, IL-1R-, BCR- and CD40-mediated immune responses in several cell types. We have demonstrated that Ubc13 deficiency 'preferentially' affects the activation of MAP kinases at least in part through induction of ubiquitination of IKKγ. Further studies using conditionally Ubc13-deficient mice may provide a new insight into other Ubc13-dependent, K63-linked, ubiquitination-related biological processes, such as those induced by DNA repair and cellular stress.

METHODS

Generation of *Ubc13*$^{fl/fl}$ mice. Genomic DNA containing *Ubc13* was isolated from a 129/Sv mouse genomic library and was characterized by restriction enzyme mapping and sequencing analysis. The targeting vector was constructed by replacement of a 3.0-kilobase fragment of *Ubc13* with a neomycin-resistance gene cassette. In addition, a 3.0-kilobase fragment of *Ubc13* genomic DNA containing exons 2, 3 and 4 was inserted between two *loxP* sites in the targeting vector pKSTKNEOLOXP, which contains a herpes simplex virus thymidine kinase gene driven by a PGK promoter. The targeting vector was transfected into embryonic stem cells (embryonic day 14.1). Colonies resistant to both G418 and ganciclovir were screened. Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F_1 progeny were

intercrossed to generate *Ubc13*$^{fl/fl}$ mice. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases of Osaka University (Osaka, Japan).

Reagents, cells and mice. LPS, poly(I:C), BLP, MALP-2 and CpG oligodeoxynucleotides were prepared as described[42]. Agonistic anti-CD40 and anti-IgM were purchased from PharMingen. B cell–activating factor of the TNF family, IL-1β and TNF were from Genzyme. Antibodies specific for the phosphorylated forms of Erk (9101), Jnk (9251), p38 (9211), IKK (3031) and IκBα (9241) were purchased from Cell Signaling. Antibodies specific for Erk (sc-94), IκBα (sc-371), TRAF6 (sc-7221), IKKγ (sc-8330), ubiquitin (sc-8017), hemagglutinin (sc-3792), histidine (sc-8036), actin (sc-8432) and NF-κBp52 (sc-298) were from Santa Cruz. Monoclonal anti-Ubc13 (37-1100) was from Zymed. Anti-IRAK-1 and anti-phospho-TAK1 have been described[49,50]. The IKKγ-deficient cell line 5R and control cell line Rat-1 (ref. 51), the IKKγ-deficient cell line 1.3E2 and control cell line 70Z/3 (ref. 40), IKK-γ-deficient MEFs[52] and MEFs homozygous for *lox*P-flanked TAK1 and expressing retroviral Cre[19] have been described. Cd19-Cre and LyzsM-Cre mice have been described[53,54].

Preparation of bone marrow macrophages. Bone marrow cells were isolated from femurs and were cultured in RPMI 1640 medium supplemented with 10% FBS and 50 ng/ml of macrophage colony-stimulating factor (R&D Systems). Medium was replaced every 2.5 d. In these conditions, monolayers of adherent cells of which more than 99% expressed surface Mac-1 were obtained. Cells were collected by incubation with 10 mM EDTA in PBS with gentle agitation and were seeded onto plates. After culture for several hours without macrophage colony-stimulating factor, cells were used as bone marrow macrophages.

Measurement of proinflammatory cytokines and electrophoretic mobility-shift assay. These assays were done as described[55].

Purification of B cells and splenic CD4$^+$ T cells. Resting B cells were isolated from splenocyte single-cell suspensions by positive selection with anti-B220 magnetic beads (Miltenyi Biotec). Cell purity was typically more than 97% for B220$^+$ cells, as assessed by flow cytometry.

Plasmids and retroviral transduction. The NF-κB-dependent reporter plasmids and expression vectors containing *Card11* have been described[55,56]. The retroviral vector pMRX encoding Cre protein and the production of retroviruses have been described[19]. At 12 h after infection, 3 μg/ml of puromycin (Invivogen) was added and selected cells were analyzed 48–72 h after infection. Hemagglutinin-tagged wild-type and K392R IKKγ constructs were generated by PCR and were cloned into pMRX retroviral vectors as described[57].

Luciferase reporter assay. This reporter assay was done with the Dual-Luciferase Reporter Assay System (Promega) as described[57].

Immunoblot, immunoprecipitation and *in vivo* ubiquitination assay. After several hours of 'starvation' in DMEM containing 0.1% FBS (to reduce background signal), cells were stimulated with various ligands for various times. Immunoblot and immunoprecipitation were done as described[57]. For detection of *in vivo* ubiquitination of IKKγ and TRAF6, cell lysates were boiled for 10 min at 90 °C in 1% SDS for removal of noncovalently attached proteins, followed by immunoprecipitation with anti-IKKγ or anti-TRAF6 in 0.1% SDS lysis buffer in the presence of protease inhibitors. Ubiquitin was detected by immunoblot anlaysis.

Cell viability assay. Purified splenic B cells (1×10^6) were stimulated with various ligands for various times. Cell viability was assessed with the MEBCYTO Apoptosis kit (MBL) and a FACSCalibur (Becton Dickinson).

Lymphocyte proliferation assay, cell cycle analysis and flow cytometry. These assays were done as described[19].

***In vivo* immunization and enzyme-linked immunosorbent assay (ELISA).** These assays were done as described[19].

Immunohistochemistry. Spleen tissue was embedded in optimum cutting temperature compound (Lab-Tek Products) and was 'flash frozen' in liquid nitrogen. Sections 5 μm in thickness were fixed in cold acetone, were air-dried and were incubated for 1 h at 25 °C in PBS containing 1% BSA, 10% normal rat serum and 5% normal goat serum. Tissue sections were then stained for 60 min at 25 °C with biotin-conjugated anti-mouse CD19 (Pharmingen) followed by streptavidin-conjugated alkaline phosphatase for 30 min, or with rat monoclonal antibody to mouse metallophilic macrophages (MCA947F; Serotec) followed by horseradish peroxidase–conjugated anti-rat. After being stained, sections were washed and were developed with the Vector Blue Alkaline Phosphatase Substrate Kit or the AEC (3-amino-9-ethylcarbazole) Substrate Kit for Horseradish Peroxidase (both from Vector), respectively.

Recombinant TRAF6-inducible *in vitro* system. This *in vitro* system was as described with slight modifications[16,17]. This used TRAF6 cDNA subcloned into pFAST-Bac-HTa (Gibco BRL) for expression in Sf9 insect cells as six-histidine–tagged proteins. Proteins were purified in accordance with the manufacturer's instructions strictly in the absence of EDTA. For detection of phosphorylation of endogenous TAK1 and IKK, cell extracts (3 mg/ml) prepared in reaction buffer (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl; after sonication) were mixed for 2 h at 30 °C with 2 mM ATP, 2 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 M wild-type ubiquitin or 0.1 M methylated ubiquitin in the presence or absence of 0.1 μM recombinant TRAF6. The reaction was terminated by the addition of SDS-PAGE sample buffer, followed by immunoblot analysis with phosphorylation-specific antibodies as described above.

Statistical analysis. We used the unpaired Student's *t*-test to determine the statistical significance of experimental data.

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

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AUTHOR CONTRIBUTIONS

All authors contributed to data analysis, experimental design, critical discussions and manuscript preparation; M.Y did all experimental studies; T.O. and Y.M. purified recombinant TRAF6; K.T. generated Ubc13-deficient mice; S.S. and S.U. prepared whole-cell extracts; T.S., N.Y. and S.Y. designed retroviral vectors; H.S. prepared antibodies; K.J.I., T.K. and O.T. played a pivotal role in discussions; and S.A. supervised all work.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c$^+$ lamina propria cells

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Toll-like receptors (TLRs) recognize distinct microbial components and induce innate immune responses. TLR5 is triggered by bacterial flagellin. Here we generated *Tlr5*$^{-/-}$ mice and assessed TLR5 function *in vivo*. Unlike other TLRs, TLR5 was not expressed on conventional dendritic cells or macrophages. In contrast, TLR5 was expressed mainly on intestinal CD11c$^+$ lamina propria cells (LPCs). CD11c$^+$ LPCs detected pathogenic bacteria and secreted proinflammatory cytokines in a TLR5-dependent way. However, CD11c$^+$ LPCs do not express TLR4 and did not secrete proinflammatory cytokines after exposure to a commensal bacterium. Notably, transport of pathogenic *Salmonella typhimurium* from the intestinal tract to mesenteric lymph nodes was impaired in *Tlr5*$^{-/-}$ mice. These data suggest that CD11c$^+$ LPCs, via TLR5, detect and are used by pathogenic bacteria in the intestinal lumen.

Toll-like receptors (TLRs) recognize a variety of pathogen-associated molecular patterns and induce innate immune responses[1]. TLRs are abundantly expressed on 'professional' antigen-presenting cells such as macrophages and dendritic cells (DCs) and serve as an important link between the innate and adaptive immune responses. So far, 13 TLRs have been identified in mammals. Among the TLR family members, TLR5 was the first to be shown to recognize a protein ligand, bacterial flagellin[2]. Bacterial flagellin is a structural protein that forms the main portion of flagella, which promote bacterial chemotaxis and bacterial adhesion to and invasion of host tissues[3]. Flagellin of *Listeria monocytogenes* and *Salmonella typhimurium* stimulates TLR5 (ref. 4). Thus, TLR5 recognizes flagellin from both Gram-positive and Gram-negative bacteria. *In vitro* studies have shown that TLR5 recognizes the conserved domain in flagellin monomers and triggers proinflammatory as well as adaptive immune responses[5]. In addition, TLR5 is expressed on the basolateral surface of intestinal epithelial cells and is thought to be key in the recognition of invasive flagellated bacteria at the mucosal surface[4]. When exposed to flagellin, human intestinal epithelial cell lines produce chemokines that induce subsequent migration of immature DCs[6]. There is high expression of TLR5 in the human lung[7], and a correlation between a common human TLR5 polymorphism and susceptibility to legionellosis has been identified[8]. Although accumulating evidence suggests that TLR5 is an important sensor for flagellated pathogens, the *in vivo* function of TLR5 is yet to be elucidated.

Here we generated *Tlr5*$^{-/-}$ mice and examined the function of TLR5 *in vivo* in the intestine. We confirmed that flagellin is a natural ligand for TLR5. Although it is known that *in vivo* administration of flagellin induces inflammatory cytokine production, it remains unclear which cell populations produce those cytokines. Because it is known that there is high expression of TLR5 in the intestine, we first isolated and examined intestinal epithelial cells (IECs). Unexpectedly, TLR5 expression in IECs was much lower than that in the whole intestine. Consistent with that, IECs did not produce inflammatory cytokines in response to flagellin. Using a new method for isolating intestinal lamina propria cells (LPCs)[9], we found that CD11c$^+$ LPCs 'preferentially' expressed TLR5 and produced inflammatory cytokines after exposure to flagellin. CD11c$^+$ LPCs sensed pathogenic flagellated bacteria via TLR5 and induced inflammatory responses. In contrast, CD11c$^+$ LPCs do not express TLR4 and did not produce proinflammatory cytokines in response to a commensal bacterium. Although TLR5 initially induced host defenses against flagellated bacteria, *Tlr5*$^{-/-}$ mice were resistant to oral *S. typhimurium* infection. The transport of *S. typhimurium* from the intestinal tract to the mesenteric

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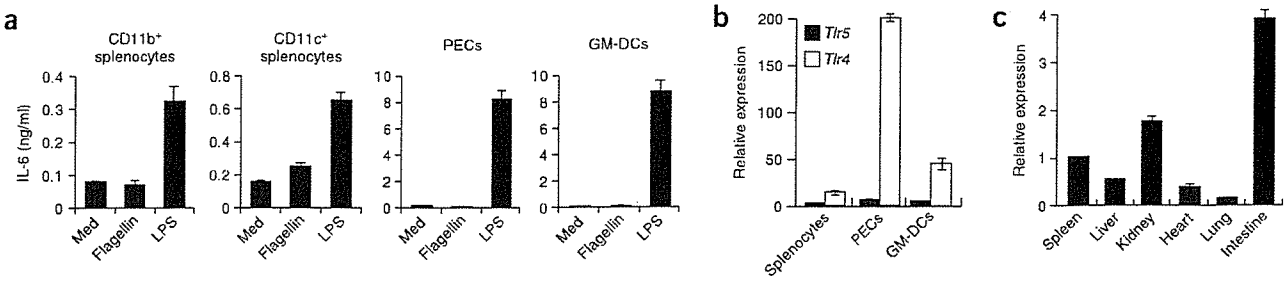

Figure 1 Macrophages and conventional DCs are hyporesponsive to flagellin. **(a)** Enzyme-linked immunosorbent assay of IL-6 production by splenic CD11b$^+$ and CD11c$^+$ cells, GM-DCs and peritoneal macrophages (PECs) from C57BL/6 mice. Cells were cultured with medium only (Med), flagellin (1 μg/ml) or LPS (100 ng/ml). **(b,c)** Quantitative real-time PCR of *Tlr5* and *Tlr4* expression in various cell types **(b)** or organs **(c)** of C57BL/6 mice. Data are mean ± s.d. of triplicate samples from one representative of three independent experiments.

lymph nodes (MLNs) was impaired in *Tlr5*$^{-/-}$ mice. These results suggest that TLR5$^+$CD11c$^+$ LPCs detect and can be used by pathogenic bacteria in the intestine.

RESULTS

Flagellin is a natural ligand for TLR5

To elucidate the physiological function of TLR5, we generated *Tlr5*$^{-/-}$ mice by gene targeting. Mouse *Tlr5* consists of one exon. We constructed the targeting vector to allow insertion of a neomycin-resistance gene cassette into that exon (**Supplementary Fig. 1**). We microinjected two correctly targeted embryonic stem clones into C57BL/6 blastcysts to generate chimeric mice. We crossed chimeric male mice with C57BL/6 female mice and monitored transmission of the mutated allele by Southern blot analysis (**Supplementary Fig. 1**). We then interbred heterozygous mice to produce offspring carrying the null mutation of *Tlr5*. *Tlr5*$^{-/-}$ mice were born at the expected mendelian ratio and showed no developmental abnormalities. To confirm the disruption of *Tlr5*, we analyzed total intestinal RNA from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice by RNA blot and detected no *Tlr5* transcripts in *Tlr5*$^{-/-}$ intestinal RNA (**Supplementary Fig. 1**).

To assess the involvement of TLR5 in the systemic production of proinflammatory cytokines in response to flagellin, we measured the concentrations of interleukin 6 (IL-6) and IL-12p40 in sera of *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice at various time points after intraperitoneal injection of purified flagellin. Although IL-6 and IL-12p40 concentrations in the serum increased within 2 h of injection in *Tlr5*$^{+/+}$ mice, their concentrations remained low even at 4 h after injection in *Tlr5*$^{-/-}$ mice (**Supplementary Fig. 1**). These results confirmed that flagellin is a natural ligand for TLR5.

Immune cell responses to flagellin

We next analyzed flagellin-mediated immune responses in macrophages and conventional DCs. We isolated CD11b$^+$ or CD11c$^+$ splenocytes, peritoneal macrophages and granulocyte-macrophage colony stimulating factor–induced bone marrow–derived DCs (GM-DCs) from *Tlr5*$^{+/+}$ mice, stimulated these cells with flagellin or the TLR4 ligand lipopolysaccharide (LPS) and measured IL-6 concentrations in cell culture supernatants (**Fig. 1a**). All cell types produced IL-6 after stimulation with LPS, but IL-6 production was not induced by stimulation with flagellin. In agreement with those results, splenocytes, peritoneal macrophages and GM-DCs had high expression of *Tlr4* but not *Tlr5* mRNA, as determined by quantitative real-time PCR (**Fig. 1b**). To identify the tissues involved in flagellin-induced production of proinflammatory cytokines, we measured *Tlr5* mRNA in the spleen, liver, kidney, heart, lung and intestine by quantitative real-time PCR and found that intestine had the highest expression of *Tlr5* mRNA (**Fig. 1c**).

TLR5 expression is confined to the basolateral surface of IECs[4]. To examine TLR5-mediated inflammatory responses in IECs, we isolated IECs from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice, stimulated them with flagellin and used cDNA microarray to examine the profile of genes induced by TLR5 stimulation (**Fig. 2**). It has been reported that flagellin induces expression of genes encoding some chemokines (such as IL-8 and CCL20) in human IEC lines[6,10]. Our analyses showed flagellin-induced expression of some genes encoding proteins involved in immune responses, such as defensin-β3, CD86, killer cell lectin-like receptor subfamily A member 6, complement component 8α and

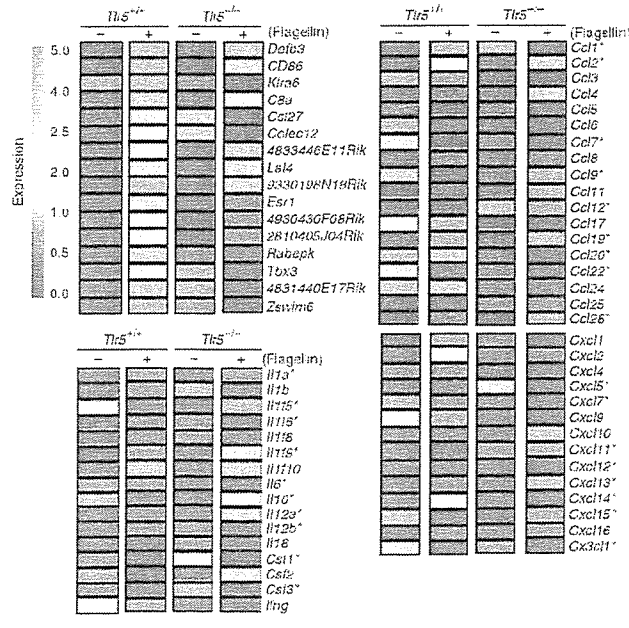

Figure 2 Gene expression induced by flagellin stimulation in IECs. Microarray analysis of IECs from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice stimulated with medium alone (–) or 1 μg/ml of flagellin (+). *, genes judged as being statistically undetectable at all time points. There is flagellin-induced expression of the genes encoding defensin-β3 (*Defb3*), CD86 (*Cd86*), killer cell lectin-like receptor subfamily A member 6 (*Klra6*), complement component 8α (*C8a*) and chemokine (C-C motif) ligand 27 (*Ccl27*) in *Tlr5*$^{+/+}$ but not *Tlr5*$^{-/-}$ IECs. Data are representative of three independent experiments.

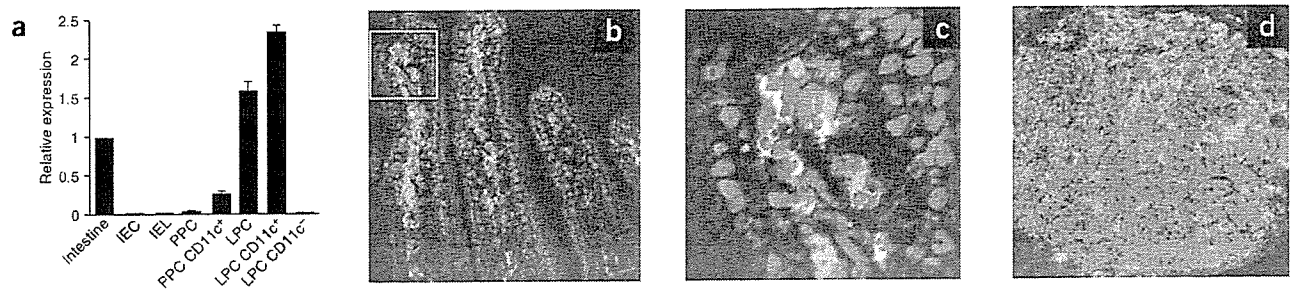

Figure 3 TLR5 is highly expressed on CD11c$^+$ LPCs. **(a)** Quantitative real-time PCR of *Tlr5* expression by the intestine (far left) and by various cell types of C57BL/6 mice. IEL, intestinal epithelial lymphocyte. Data are mean ± s.d. of triplicate samples from one representative of three independent experiments. **(b–d)** Confocal microscopy of frozen tissue sections of small intestine **(b,c)** and Peyer's patch **(d)** of C57BL/6 mice, fixed and stained with antibodies specific for CD11c (red) and TLR5 (green). Image in **c** is an enlargement of the boxed area in **b**. Original magnification ×400 **(b,d)** and ×1,000 **(c)**. Data are from one of three representative experiments.

chemokine (C-C motif) ligand 27 in *Tlr5*$^{+/+}$ but not *Tlr5*$^{-/-}$ IECs. However, most genes encoding chemokines were not induced by flagellin, even in *Tlr5*$^{+/+}$ IECs, and flagellin did not induce the expression of any genes encoding proinflammatory cytokines in *Tlr5*$^{+/+}$ IECs. We confirmed that *Tlr5*$^{+/+}$ IECs did not produce proinflammatory cytokine protein after flagellin stimulation (data not shown). There was much less *Tlr5* mRNA in IECs than in the entire small intestine (**Fig. 3a**).

Because TLR5 expression was low in IECs but high in the entire small intestine, we hypothesized that TLR5 must be 'preferentially' expressed in other intestinal cell types. We measured *Tlr5* mRNA in Peyer's patch cells (PPCs), intestinal epithelial lymphocytes and LPCs (**Fig. 3a**). There was high expression of *Tlr5* mRNA in LPCs, but *Tlr5* mRNA expression in intestinal epithelial lymphocytes and PPCs was lower than that in the entire small intestine. DCs are a dominant antigen-presenting cell in the lamina propria of mouse small bowel[11]. Therefore, we separated CD11c$^+$ cells from LPCs and PPCs and measured expression of *Tlr5* mRNA. We detected considerable *Tlr5* mRNA in CD11c$^+$ LPCs but not CD11c$^-$ LPCs. CD11c$^+$ PPCs had less *Tlr5* mRNA than did CD11c$^+$ LPCs. Next we examined the localization of TLR5 protein in the small intestine by immunohistochemistry. In agreement with the mRNA expression data, there was high expression of TLR5 on intestinal CD11c$^+$ LPCs (**Fig. 3b,c**) but not on PPCs (**Fig. 3d**). Thus, TLR5 is expressed specifically on CD11c$^+$ LPCs in the small intestine.

Next we assessed the effect of flagellin stimulation on CD11c$^+$ LPCs. *Tlr5*$^{+/+}$ but not *Tlr5*$^{-/-}$ CD11c$^+$ LPCs produced IL-6 and IL-12p40 in response to flagellin (**Fig. 4**, top). However, CD11c$^+$ LPCs did not produce large amounts of tumor necrosis factor after stimulation with flagellin and failed to produce any cytokines after LPS stimulation.

Antigen-presenting cells in Peyer's patches have been extensively characterized[12]. Peyer's patches contain unusual subsets of DCs that are important in the generation of regulatory T cells and the induction of oral tolerance[12,13]. These Peyer's patch DCs produce IL-10 in response to inflammatory stimulations such as LPS[14]. Consistent with their low expression of *Tlr5* mRNA (**Fig. 3a**), CD11c$^+$ PPCs did not produce inflammatory cytokines after stimulation with flagellin (**Fig. 4**, bottom). However, CD11c$^+$ PPCs produced IL-6 and IL-10 in response to LPS. In contrast, neither *Tlr5*$^{+/+}$ nor *Tlr5*$^{-/-}$ CD11c$^+$ LPCs produced IL-10 in response to flagellin, suggesting that in CD11c$^+$ LPCs, TLR5 signaling induces inflammatory responses but not tolerance (**Fig. 4**).

To comprehensively examine TLR5-mediated innate immune responses in the small intestine, we obtained RNA from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ LPCs stimulated for 4 h with flagellin and hybridized the RNA to cDNA microarrays (**Fig. 5**). Several transcripts were substantially upregulated at 4 h after flagellin stimulation in *Tlr5*$^{+/+}$ but not *Tlr5*$^{-/-}$ LPCs. These included genes encoding proinflammatory molecules such as cytokines, cytokine receptors, chemokines, signaling molecules, prostanoids, prostanoid synthetase and secretary antimicrobial peptides (**Fig. 5**, top). Genes associated with cellular adhesion,

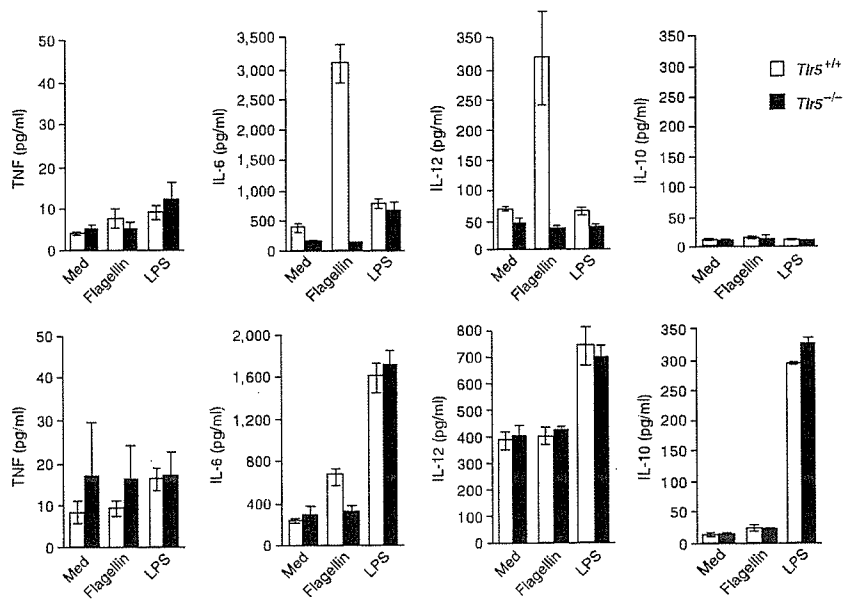

Figure 4 TLR5-mediated CD11c$^+$ LPC cytokine production. Enzyme-linked immunosorbent assay of cytokine production by CD11c$^+$ LPCs (top) and PPCs (bottom) from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice. Cells were cultured with medium only, flagellin (1 μg/ml) or LPS (100 ng/ml). Data are mean ± s.d. of triplicate samples from one representative of three independent experiments.

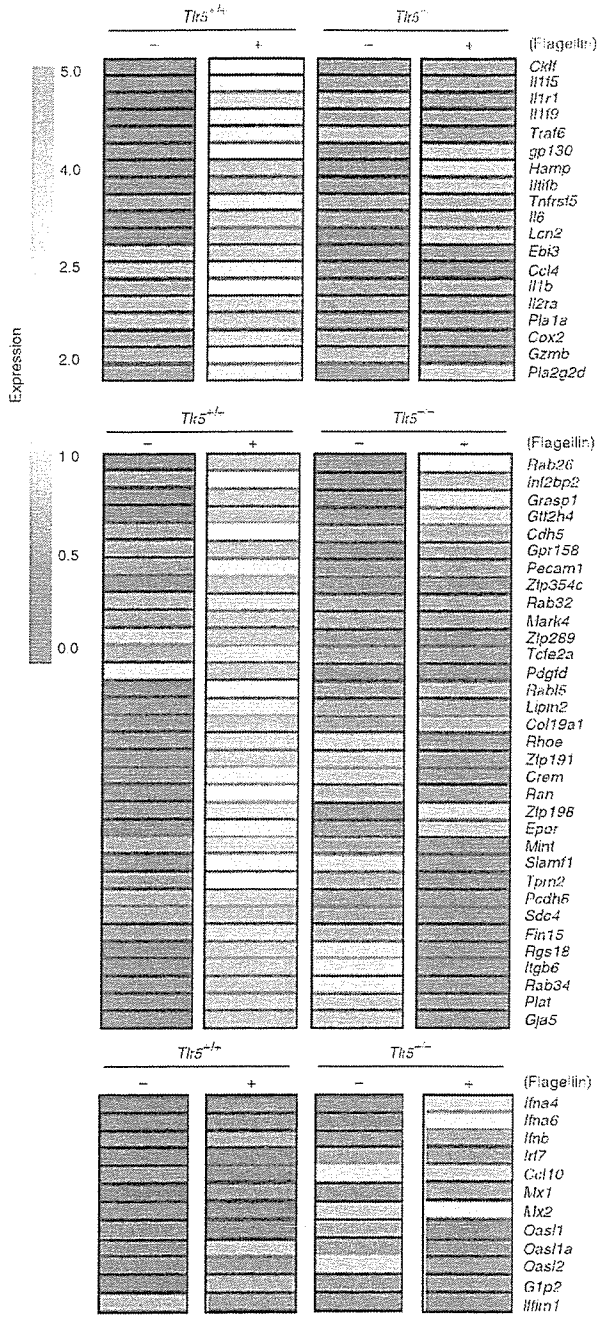

Figure 5 Flagellin-induced gene expression in CD11c$^+$ LPCs. Microarray analysis of CD11c$^+$ LPCs from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice left unstimulated (–) or stimulated with 1 μg/ml of flagellin (+). Upregulated genes encode cytokines (*Il6, Il1f5, Il1f9, Il1b, Ebi3* and *Il1fb*), cytokine receptors (*Tnfrsf5, Il1r1* and *Il2ra*), chemokines (*Cklf* and *Ccl4*), signaling molecules (*Traf6* and *gp130*), prostanoids (*Pla1a* and *Pla2g2d*), prostanoid synthetase (*Cox2*) and secretary antimicrobial peptides (*Hamp, Lcn* and *Gzmb*; top), as well as molecules associated with cellular adhesion, cytoskeletal organization, intracellular transport, vesicle fusion and transcription (middle). Data are representative of three independent experiments.

cytoskeletal organization, intracellular transport, vesicle fusion and transcription were also upregulated by flagellin stimulation (**Fig. 5**, middle). In contrast, interferon and interferon-inducible genes were not induced in response to flagellin in either *Tlr5*$^{+/+}$ or *Tlr5*$^{-/-}$ LPCs (**Fig. 5**, bottom).

CD11c$^+$ LPCs detect pathogenic bacteria via TLR5

CD11c$^+$ LPCs produced IL-6 and IL-12p40 in response to flagellin but not LPS stimulation. CD11c$^+$ LPCs produced similar amounts of IL-6 when stimulated through TLR2 or TLR9 (**Supplementary Fig. 2** online), suggesting that LPS signaling is suppressed specifically in CD11c$^+$ LPCs. Therefore, we measured TLR4 and TLR5 in CD11c$^+$ LPCs and CD11c$^+$ splenic cells (SPCs; **Fig. 6a**). *Tlr4* expression was high and *Tlr5* expression was low in CD11c$^+$ SPCs. In contrast, *Tlr4* expression was low and *Tlr5* expression was high in CD11c$^+$ LPCs.

As CD11c$^+$ LPCs and SPCs had different expression profiles for TLR4 and TLR5, we assessed their responses to commensal and pathogenic bacteria. We isolated CD11c$^+$ LPCs and CD11c$^+$ SPCs from wild-type, *Tlr4*$^{-/-}$ and *Tlr5*$^{-/-}$ mice and measured IL-6 production induced by stimulation with heat-killed commensal Gram-negative bacteria (*Enterobacter cloacae*) and pathogenic Gram-negative bacteria (*S. typhimurium*; **Fig. 6b**). Wild type and *Tlr5*$^{-/-}$ CD11c$^+$ SPCs produced copious IL-6 in response to both *E. cloacae* and *S. typhimurium*. However, *Tlr4*$^{-/-}$ CD11c$^+$ SPCs produced less IL-6 than did wild-type or *Tlr5*$^{-/-}$ CD11c$^+$ SPCs, suggesting that CD11c$^+$ SPCs induce innate immune responses to Gram-negative bacteria mainly via TLR4. Wild-type and *Tlr4*$^{-/-}$ CD11c$^+$ LPCs produced copious IL-6 in response to *S. typhimurium*. In contrast, *Tlr5*$^{-/-}$ CD11c$^+$ LPCs produced little IL-6 after stimulation with *S. typhimurium*. We further assessed the response of CD11c$^+$ LPCs with a mutant strain of *S. typhimurium* that lacks the *fliA* gene and therefore does not produce flagella[15]. Wild-type and *Tlr5*$^{-/-}$ CD11c$^+$ LPCs were hyporesponsive to this *fliA* mutant (compared with their response to wild-type *S. typhimurium*), suggesting that CD11c$^+$ LPCs induce immune responses after recognizing flagellin of *S. typhimurium*. Unlike wild-type CD11c$^+$ SPCs, wild-type CD11c$^+$ LPCs produced a relatively small amount of IL-6 after stimulation with *E. cloacae*. These data suggest that CD11c$^+$ LPCs detect pathogenic flagellated bacteria and induce innate immune responses via TLR5.

S. typhimurium uses TLR5 for systemic infection

To investigate whether TLR5 has a specific function in fighting bacterial infection in the intestine, we orally infected *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice with *S. typhimurium*. Unexpectedly, when assessed on a mixed genetic background (C56BL/6 × 129Sv, F2), all *Tlr5*$^{-/-}$ mice survived a dose of *S. typhymurium* that was lethal for *Tlr5*$^{+/+}$ mice (**Fig. 7a**, left). Next we assessed the resistance of *Tlr5*$^{-/-}$ mice backcrossed onto the C57BL/6 genetic background. Although wild-type C57BL/6 mice are resistant to oral *S. typhymurium* infection, *Tlr5*$^{-/-}$ C57BL/6 mice background were significantly more resistant, even at an extremely high dose (5×10^8 bacteria; **Fig. 7a**, right). These results indicate that *Tlr5*$^{-/-}$ mice were resistant regardless of their genetic background. When we challenged mice with *S. typhimurium* by intraperitoneal injection, we noted no significant difference in the survival of *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice (**Fig. 7b**). Furthermore, we recovered fewer bacteria from the livers and spleens of *Tlr5*$^{-/-}$ mice than *Tlr5*$^{+/+}$ mice 4 d after oral infection (**Fig. 7c**). At 48 h after oral infection, *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice had the same number of *S. typhimurium* in Peyer's patches and LPCs. However, *Tlr5*$^{-/-}$ mice had fewer bacteria in MLNs than did *Tlr5*$^{+/+}$ mice (**Fig. 7d**). In addition, the proportion of *S. typhimurium*–laden CD11c$^+$ cells in MLNs of *Tlr5*$^{-/-}$ mice was smaller than that in *Tlr5*$^{+/+}$ mice (**Supplementary Fig. 3** online). To further determine whether the transport

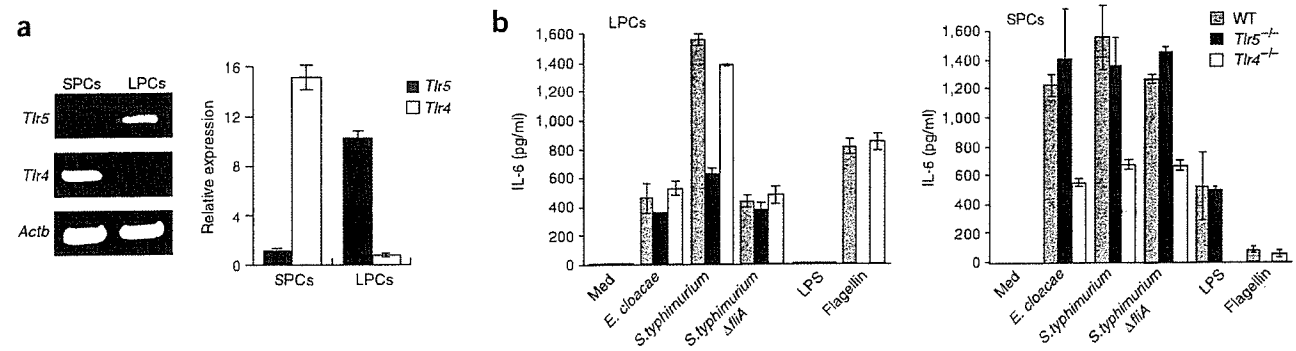

Figure 6 CD11c$^+$ LPCs detect pathogenic bacteria via TLR5. **(a)** Quantitative real-time PCR of *Tlr5* and *Tlr4* expression in CD11c$^+$ SPCs and CD11c$^+$ LPCs of C57BL/6 mice. *Actb* encodes β-actin (loading control). Graphed data are mean ± s.d. of triplicate samples from one representative of three independent experiments. **(b)** Enzyme-linked immunosorbent assay of cytokine production by CD11c$^+$ SPCs and CD11c$^+$ LPCs from wild-type (WT), *Tlr4*$^{-/-}$ and *Tlr5*$^{-/-}$ mice, cultured with medium along (Med) or various stimuli (horizontal axes). Δ*fliA*, mutant strain lacking *fliA*. Data are mean ± s.d. of triplicate samples from one representative of three independent experiments.

of *S. typhimurium* from intestinal tract to MLNs was impaired in *Tlr5*$^{-/-}$ mice, we challenged *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice in a surgically isolated intestinal loop with *S. typhimurium* expressing green fluorescent protein (**Supplementary Fig. 4** online). We collected MLNs 24 h after infection. Staining showed that *Tlr5*$^{-/-}$ mice had fewer *S. typhimurium*–laden CD11c$^+$ cells (one to two cells per longitudinal slice of MLN) than did *Tlr5*$^{+/+}$ mice (about ten cells per longitudinal slice of MLN). Furthermore, no cells except CD11c$^+$ cells contained *S. typhimurium* in the infected MLNs. Thus, the impairment of transport of *S. typhimurium* from the intestinal tract to MLNs may lead to a delay in the establishment of systemic infection in *Tlr5*$^{-/-}$ mice.

DISCUSSION

Although TLR5 has been identified as a receptor of flagellin *in vitro*, its *in vivo* function has remained unclear. Addressing the function of TLR5 in innate immunity has been difficult, because unlike other TLR family members, TLR5 is not expressed in mouse spleen cells, peritoneal macrophages or GM-DCs. Using a new method of isolating LPCs with high viability[9], we found that TLR5 is specifically expressed on CD11c$^+$ LPCs in mouse intestine. Although it has long been known that DCs are present in the lamina propria under the villus epithelium and take up antigens from the intestine[16], their functions and properties in the intestine were unknown. CD11c$^+$ LPCs elicited the secretion of various mediators, including inflammatory cytokines, chemokines, antimicrobial peptides and tissue remodeling kinases, in response to flagellin. Thus, we have shown here that immune responses are induced in CD11c$^+$ LPCs via TLR5.

Two points regarding the function of CD11c$^+$ LPCs in relation to TLR5 came to light as a result of our analyses. One was the cytokine profile of CD11c$^+$ LPCs stimulated with flagellin. The gut is continuously exposed to food antigens and many commensal bacteria. Tolerance to beneficial antigens seems to be controlled by mucosal DCs[17]. These DCs stimulate the activity of regulatory T cells, which are potent suppressors of T cell responses. A CD11c^{lo}CD45RB^{hi} DC subset that produces IL-10 has been shown to specifically promote suppressive functions in regulatory T cells[14]. Peyer's patches contain DCs that produce IL-10 after inflammatory stimulation and thereby promote oral tolerance[12,13]. Whereas CD11c$^+$ PPCs induced IL-10 in response to LPS, flagellin-stimulated CD11c$^+$ LPCs did not produce IL-10, but instead produced IL-6 and IL-12, suggesting that CD11c$^+$ LPCs have a tendency to induce inflammatory responses rather than tolerance when stimulated with flagellin. However, it has been

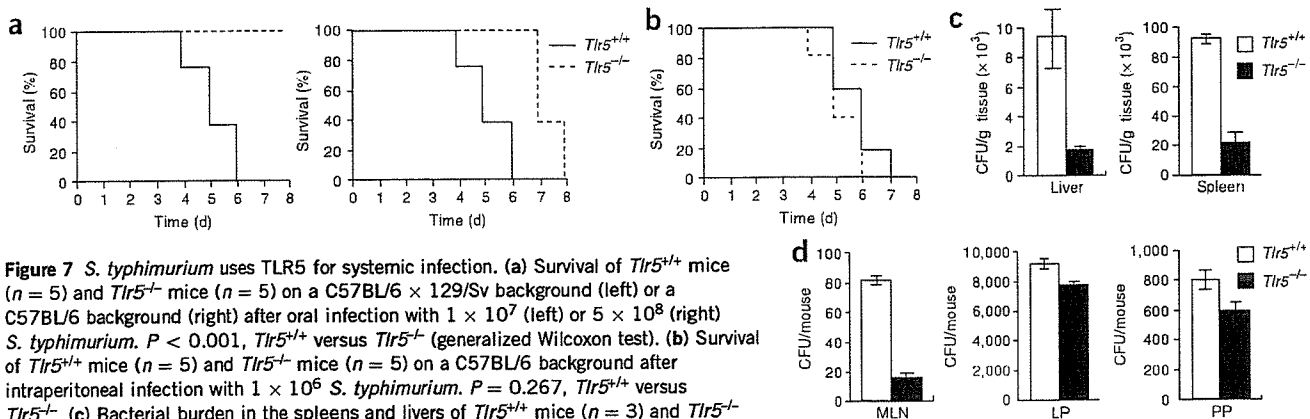

Figure 7 *S. typhimurium* uses TLR5 for systemic infection. **(a)** Survival of *Tlr5*$^{+/+}$ mice ($n = 5$) and *Tlr5*$^{-/-}$ mice ($n = 5$) on a C57BL/6 × 129/Sv background (left) or a C57BL/6 background (right) after oral infection with 1×10^7 (left) or 5×10^8 (right) *S. typhimurium*. $P < 0.001$, *Tlr5*$^{+/+}$ versus *Tlr5*$^{-/-}$ (generalized Wilcoxon test). **(b)** Survival of *Tlr5*$^{+/+}$ mice ($n = 5$) and *Tlr5*$^{-/-}$ mice ($n = 5$) on a C57BL/6 background after intraperitoneal infection with 1×10^6 *S. typhimurium*. $P = 0.267$, *Tlr5*$^{+/+}$ versus *Tlr5*$^{-/-}$. **(c)** Bacterial burden in the spleens and livers of *Tlr5*$^{+/+}$ mice ($n = 3$) and *Tlr5*$^{-/-}$ mice ($n = 3$) on a C57BL/6 background 96 h after oral infection with 5×10^8 *S. typhimurium*. **(d)** Bacterial burden in the MLNs, PPCs and LPCs of *Tlr5*$^{+/+}$ mice ($n = 5$) and *Tlr5*$^{-/-}$ mice ($n = 5$) on C57BL/6 background 48 h after oral infection with 5×10^8 *S. typhimurium*. CFU, colony-forming units. Data are one representative of three independent experiments (**a,b**) or are mean ± s.d. of triplicate samples from one representative of three independent experiments (**c,d**).

reported that DCs in the lamina propria are involved in oral tolerance induction[18,19]. Further study will help elucidate CD11c$^+$ LPC-mediated regulation of tolerance and host defense.

The second notable point was that TLR4 expression was very low in CD11c$^+$ LPCs. Most commensal bacteria in intestine are Gram-negative anaerobic rod bacteria, which contain LPS in their cell wall. It has been shown that CD11c$^+$ LPCs extend their dendrites to sample bacteria in the intestinal lumen[20]. Although the mechanism of bacterial sampling by CD11c$^+$ LPCs was fully analyzed, it has remained unclear how host intestinal mucosa remains tolerant to commensal bacteria and discriminates between commensal and pathogenic bacteria. Low expression of TLR4 may allow CD11c$^+$ LPCs to avoid inducing inappropriate immune responses after exposure to commensal bacteria. Instead, CD11c$^+$ LPCs induced inflammatory responses after exposure to pathogenic flagellated bacteria mainly via TLR5. Some commensal bacteria also have flagella, but CD11c$^+$ LPCs did not respond vigorously to those bacteria. In addition, it has been reported that some commensal bacteria, such as α- and ε-proteobacteria, change the TLR5-recognition site of flagellin without losing flagellar motility[21]. Furthermore, some commensal bacteria suppress flagellin expression in stable host environments[12]. Therefore, unlike pathogenic bacteria, commensal bacteria may have mechanisms to escape TLR5-mediated host detection.

Other TLR family members, such as TLR2 and TLR4, also recognize bacterial components. The importance of TLR2 and TLR4 in host defense against various bacteria has been demonstrated with *Tlr2*$^{-/-}$ mice and *Tlr4*$^{-/-}$ mice. In particular, C3H/HeJ mice, which express a mutant form of TLR4, are highly susceptible to intraperitoneal infection by *S. typhimurium*[1]. Because TLR5 is highly expressed exclusively in the intestine, we predicted that no there would be no substantial difference in the survival of *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice after intraperitoneal infection. Instead, we predicted that disruption of *Tlr5* would render mice more susceptible to oral *S. typhimurium* infection, because stimulation of TLR5 induced the production of proinflammatory cytokines in CD11c$^+$ LPCs. The resistance of *Tlr5*$^{-/-}$ mice to oral *S. typhimurium* infection was unexpected. *Tlr5*$^{-/-}$ mice survived longer than *Tlr5*$^{+/+}$ mice because of impaired transport of *S. typhimurium* from the intestinal tract to the liver and spleen. We believe that this unexpected result is closely related to specific pathogenesis of salomonella. Most reports have indicated that *S. typhimurium* are captured by subepithelial DCs after transport through M cells in Peyer's patches[22] or by intraepithelial DCs that send protrusions into the lumen of the small intestine[23]. After being internalized, *S. typhimurium* actively modulates host vesicular trafficking pathways to avoid delivery to lysosomes and to establish a specialized replicative niche[24]. Bacteria-laden DCs undergo maturation and migrate to T cell zones of Peyer's patches or draining MLNs[12]. These mature DCs are also thought to be responsible for the dissemination of *S. typhimurium* through the bloodstream to the liver and spleen[12,25]. The uptake of *S. typhimurium* in Peyer's patches and LPCs was the same in *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice. Furthermore, the uptake of *S. typhimurium* was the same in *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ CD11c$^+$ LPCs *in vitro* (data not shown). However, there were many fewer bacteria in MLNs of *Tlr5*$^{-/-}$ mice than in *Tlr5*$^{+/+}$ mice, suggesting that the transport of *S. typhimurium* from lamina propria to MLNs was impaired. As *S. typhimurium* could not fully activate and mature *Tlr5*$^{-/-}$ CD11c$^+$ LPCs, migration of *S. typhimurium*–laden CD11c$^+$ LPCs from the periphery to circulation may be inefficient in *Tlr5*$^{-/-}$ mice. In support of that idea, there were many fewer *S. typhimurium*–laden CD11c$^+$ cells in *Tlr5*$^{-/-}$ mice than in *Tlr5*$^{+/+}$ mice after infection. Although TLR5 on CD11c$^+$ LPCs initially sense flagellated pathogenic bacteria to induce host defense, facultative intracellular pathogens such as *S. typhimurium* may use CD11c$^+$ LPCs as carriers for systemic infection. Further study will be needed to clarify the mechanism of systemic *S. typhimurium* infection, through the generation of a specific marker for CD11c$^+$ LPCs or a technique to specifically effect depletion of these cells. Finally, our work is likely to open new therapeutic perspectives. New methods that target TLR5 on CD11c$^+$ LPCs would be useful for mucosal adjuvant immune therapies.

METHODS

Mice, reagents and bacteria. C57BL/6 mice were purchased from CLEA Japan. *Tlr4*$^{-/-}$ mice have been described[26]. *Tlr5*$^{-/-}$ mice are described in the **Supplementary Methods** online. All animal experiments were done with an experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Research Institute for Microbial Diseases at Osaka University (Osaka, Japan). LPS from *Salmonella minnesota* Re595 was prepared with a phenol–chloroform–petroleum ether extraction procedure and purified flagellin was a gift from A. Aderem (Institute for Systems Biology, Seattle, Washington). *Salmonella enteritica* serovar typhimurium SR-11 x3181 and x3181 *fliA*::Tn*10* bacteria were provided by the Kitasato Institute for Life Science (Kitasato, Japan)[15,27]. *E. cloacae* was isolated from a healthy human volunteer and was identified by The Research Foundation for Microbial Diseases of Osaka University.

Cells. The preparation of GM-DCs and peritoneal macrophages has been described[28]. For the preparation of splenic macrophages and DCs, spleens were cut into small fragments and were incubated for 20 min at 37 °C with RPMI 1640 medium containing 400 U/ml of collagenase (Wako) and 15 μg/ml of DNase (Sigma). For the last 5 min, 5 mM EDTA was added. Single-cell suspensions were prepared after red blood cell lysis, and macrophages and DCs were positively selected with microbeads coated with antibody to CD11b (anti-CD11b) and anti-CD11 (Miltenyi), respectively. Intestinal lymphocytes and epithelial cells were isolated by a published protocol[9]. CD11c$^+$ cells from small intestine lamina propria and Peyer's patches were isolated by a published protocol[9].

Measurement of proinflammatory cytokines. GM-DCs, peritoneal macrophages, CD11b$^+$ splenocytes, CD11c$^+$ splenocytes and CD11c$^+$ LPCs were cultured in 96-well plates (5×10^4 cells/well) with LPS (100 ng/ml) or flagellin (1 μg/ml). The concentrations of tumor necrosis factor, IL-6, IL-12p40 and IL-10 in culture supernatants were measured by the Bio-Plex system (Bio-Rad) following the manufacturer's instructions.

PCR. RNA (1 μg) was reverse-transcribed with Superscript2 (Invitrogen) according to the manufacturer's instructions with random hexamers as primers. PCR used the primer pairs in **Supplementary Table 1** online and Taq polymerase (Takara Shuzo). After being incubated at 95 °C for 10 min, products were amplified by 25 cycles of 97 °C (30 s), 57 °C (30 s) and 72 °C (30 s). Products were analyzed by agarose gel electrophoresis. Quantitative real-time PCR was done with a final volume of 25 μl containing cDNA amplified as described above, 2x PCR Master Mix (Applied Biosystems) and primers for 18S rRNA (Applied Biosystems) as an internal control or primers specific for *Tlr4* or *Tlr5* (Assay on Demand), using a 7700 Sequence Detector (Applied Biosystems). After being incubated at 95 °C for 10 min, products were amplified by 35 cycles of 95 °C (15 s), 60 °C (60 s) and 50 °C (120 s).

Microarray analysis. IECs and LPCs collected from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice were left untreated or were treated for 4 h with flagellin (1 μg/ml). Total RNA was extracted with an RNeasy kit (Qiagen) and was purified with an Oligotex mRNA Kit (Pharmacia). Fragmented and biotin-labeled cDNA was synthesized from 100 ng purified mRNA with the Ovation Biotin System (Nugen) according to the manufacturer's protocol. The cDNA was hybridized to Affymetrix Murine Genome 430 2.0 microarray chips (Affymetrix) according to the manufacturer's instructions. Hybridized chips were stained and washed and were scanned with a GeneArray Scanner (Affymetrix). Microarray Suite software (Version 5.0, Affymetrix) and GeneSpring software (Silicon Genetics) were used for data analysis.

Immunofluorescence. Biotinylated monoclonal anti-mouse CD11c (HL3; Pharmingen) and anti-TLR5 (AP1505a; Abgent) were applied overnight at 4 °C to sections cut from frozen intestinal tissue. Samples were washed and then were incubated for 2 h at 25 °C with streptavidin–Alexa Fluor 594 (S-32356; Molecular Probes) and Alexa Fluor 488–chicken anti–rabbit IgG (A-21441; Molecular Probes). Staining was analyzed with a Radiance2100 laser-scanning confocal microscope (Bio-Rad). The intestinal loop assay is described in the **Supplementary Methods**.

Bacterial infection. *S. typhimurium* was grown in Luria-Bertani medium without shaking at 37 °C. The concentration of bacteria was determined by the absorbance at 600 nm. Bacteria were injected orally or intraperitoneally into 8-week-old mice. For determination of the bacterial burden in livers and spleens, LPCs, PPCs and MLNs were lysed with 0.01% Triton-X100. Serial dilutions of lysates were plated on Luria-Bertani agar plates and colonies were counted after overnight incubation at 37 °C.

Statistics. Kaplan-Meier plots and log-rank tests were used to assess the survival differences of control and mutant mice after bacterial infection.

Accession code. GEO: microarray data, GSE5119.

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

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AUTHOR CONTRIBUTIONS

S.U. and M.H.J. did most of the experiments to characterize mouse phenotypes; N.C. helped with the quantitative PCR, microarray analysis, isolation of cells and enzyme-linked immunosorbent assays; Z.G. helped to isolate cells and with immunostaining and did the surgical operations for the intestinal loop assay; Y.K. helped with analysis of microarray data; M.Y. helped to generate *Tlr5*$^{-/-}$ mice; H.K. helped with the enzyme-linked immunosorbent assays; N.S. helped to isolate cells; H.M. provided *S. typhimurium* and provided instructions for infection experiments; H.K. helped with the infection experiments; H.H. helped to generate *Tlr5*$^{-/-}$ mice; C.C. helped with the infection experiments; T.K., K.J.I. and O.T. provided advice for the experiments; M.M. provided advice for the experiments and manuscript; K.T. helped to generate *Tlr5*$^{-/-}$ mice and to design experiments; and S.A. designed all the experiments and prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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MyD88-Dependent Signaling for IL-15 Production Plays an Important Role in Maintenance of CD8$\alpha\alpha$ TCR$\alpha\beta$ and TCR$\gamma\delta$ Intestinal Intraepithelial Lymphocytes[1]

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Interaction between commensal bacteria and intestinal epithelial cells (i-ECs) via TLRs is important for intestinal homeostasis. In this study, we found that the numbers of CD8$\alpha\alpha$ TCR$\alpha\beta$ and TCR$\gamma\delta$ intestinal intraepithelial lymphocytes (i-IELs) were significantly decreased in MyD88-deficient (−/−) mice. The expression of IL-15 by i-ECs was severely reduced in MyD88$^{-/-}$ mice. Introduction of IL-15 transgene into MyD88$^{-/-}$ mice (MyD88$^{-/-}$ IL-15 transgenic mice) partly restored the numbers of CD8$\alpha\alpha$ TCR$\alpha\beta$ and TCR$\gamma\delta$ i-IELs. The i-IEL in irradiated wild-type (WT) mice transferred with MyD88$^{-/-}$ bone marrow (BM) cells had the same proportions of i-IEL as WT mice, whereas those in irradiated MyD88$^{-/-}$ mice transferred with WT BM cells showed significantly reduced proportions of CD8$\alpha\alpha$ TCR$\alpha\beta$ and TCR$\gamma\delta$ i-IELs, as was similar to the proportions found in MyD88$^{-/-}$ mice. However, irradiated MyD88$^{-/-}$ IL-15 transgenic mice transferred with WT BM cells had increased numbers of CD8$\alpha\alpha$ TCR$\alpha\beta$ and TCR$\gamma\delta$ subsets in the i-IEL. These results suggest that parenchymal cells such as i-ECs contribute to the maintenance of CD8$\alpha\alpha$ TCR$\alpha\beta$ and $\gamma\delta$ i-IELs at least partly via MyD88-dependent IL-15 production. *The Journal of Immunology,* 2006, 176: 6180–6185.

Intestinal intraepithelial lymphocytes (i-IELs),[3] which are located at the basolateral surfaces of intestinal epithelial cells (i-ECs), comprise unique T cell populations that include CD4$^-$/CD8$\alpha\alpha^+$ T cells expressing TCR$\alpha\beta$ or TCR$\gamma\delta$ and exhibit non-MHC-restricted cytotoxicity (1–3). The interaction of i-ECs and i-IELs through E-cadherin/integrin $\alpha_E\beta_7$ is important for the homing and maintenance of i-IELs (4). We previously showed that i-IELs recognized and eliminated effete i-ECs for homeostatic regulation of intestinal epithelia (5, 6). It has been reported that i-IELs, especially TCR$\gamma\delta$ i-IELs, play an important role in the regulation of generation and differentiation of i-ECs at crypts (7, 8). Taken together, results of previous studies suggest that mutual interaction of i-IELs and i-ECs is important for homeostasis of intestinal epithelia.

IL-15 is a cytokine that resembles IL-2 in its biological activity, stimulating macrophages (Mϕ), NK cells, TCR$\gamma\delta$ T cells, and B cells to proliferate, secrete cytokines, exhibit increased cytotoxicities, and produce Ab (9–12). IL-15 was found to be produced only by limited populations of cells, such as activated monocyte/Mϕ and epithelial cells, but not by activated T cells (13, 14). We previously reported that CD8$\alpha\alpha$ i-IELs proliferate preferentially in response to exogenous IL-15 (15). It has been shown that mice deficient in the *IL-15* or *IL-15R*α gene had reduced numbers of CD8$\alpha\alpha$ and/or TCR$\gamma\delta$ i-IELs (16, 17). Taken together, results of these studies suggest that IL-15 is involved in the development and proliferation of CD8$\alpha\alpha^+$ T cells in i-IELs.

It is widely accepted that intestinal microflorae play an important role in the maintenance of homeostasis of the intestinal microenvironment by inhibiting colonization by many pathogens and stimulating the growth of beneficial microorganisms. It has been reported recently that intestinal microflorae play an important role in maintaining i-ECs via TLRs (18). TLRs are a group of pattern recognition receptors that cooperate in recognizing a series of pathogens by binding to the pathogen-associated molecular patterns (19). After binding to their ligands, most of TLRs induce a series of intracellular signal transductions through MyD88, an adaptor protein for transcriptional activation of cytokine genes (20, 21). We and others previously reported that TLR signaling played important roles in activation of IL-15 transcription in LPS-stimulated Mϕ and virus-infected cell lines (22, 23). Thus, it is hypothesized that TLR signaling for IL-15 production via microflorae is involved in interaction of i-EC and i-IEL for maintaining homeostasis of the intestinal microenvironment.

In the present study, we found that the CD8$\alpha\alpha$ TCR$\alpha\beta$ and TCR$\gamma\delta$ i-IELs were selectively decreased in *MyD88*-deficient (−/−) mice, accompanied with impaired IL-15 expression by i-ECs. Introduction of IL-15 transgene into MyD88$^{-/-}$ mice was able to restore the numbers of CD8$\alpha\alpha$ TCR$\alpha\beta$ and TCR$\gamma\delta$ i-IELs, albeit partly. The experiments with bone marrow (BM) chimeras revealed that radioresistant parenchymal cells played an important role in MyD88-dependent maintenance of the i-IELs. We concluded that MyD88-dependent signaling was involved in interaction of i-EC and i-IEL for maintaining homeostasis of the intestinal microenvironment at least partly via IL-15 production.

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[3] Abbreviations used in this paper: i-IEL, intestinal intraepithelial lymphocyte; BM, bone marrow; i-EC, intestinal epithelial cell; Mϕ, macrophage; Tg, transgenic; WT, wild type.

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