おわりに

古代エジプト時代から，結核という病気が存在していたことが記述されています．結核は産業革命後の都市化に伴い大流行してきました．結核の流行は常に社会のあり様と深く関係してきています[13]．HIV の蔓延，戦争，自然災害，人口変動，多剤耐性結核の流行によっては結核が大きな社会問題となる可能性もあります．欧米先進国においては，移民，難民，ホームレス者，不安定就労者などの人々の結核問題は難しい課題となっています．我が国においても，大都市部の不安定就労者，日雇い労働者，ホームレス者の結核問題にどう対応できるかが問われています．世界の結核問題が解決されなければ，先進諸国における結核対策も終焉しないことは欧米諸国の結核再興の歴史から示されています．我が国の結核対策については，国内では既感染者が多い状況がしばらく続いていますし，また国外のアジアの国々の結核罹患率が高い状況にあることからまだ手をゆるめることができません．我が国の結核予防法は 2004 年に改正され，さらに 2006 年に改正されることになっています．世界的にも，また我が国においても結核はまだまだ日常診療の場で忘れてはならない感染症であることを認識しておく必要があります．

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nature immunology

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.

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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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Received 28 March; accepted 13 June; published online 9 July 2006; doi:10.1038/ni1362

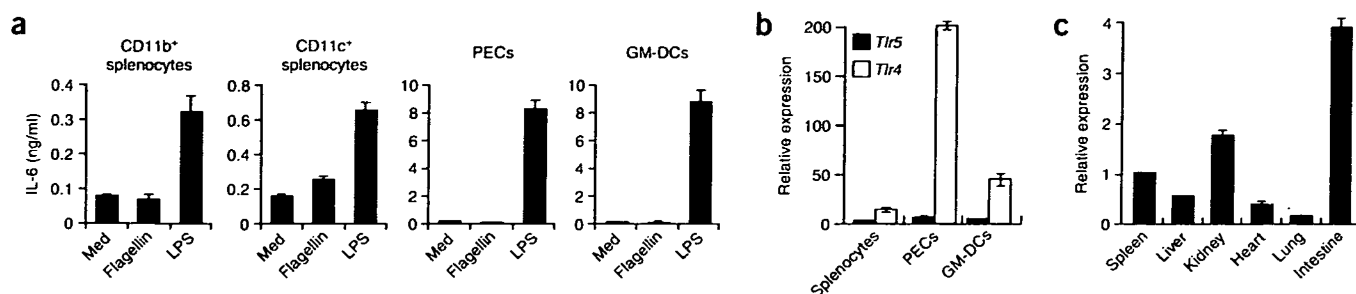

Figure 1 Macrophages and conventional DCs are hyporesponsive to flagellin. **(a)** Enzyme-linked immunosorbent assay of IL-6 production by splenic CD11b$^+$ and CD11c$^+$ cells, GM-DCs and peritoneal macrophages (PECs) from C57BL/6 mice. Cells were cultured with medium only (Med), flagellin (1 μg/ml) 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.

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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

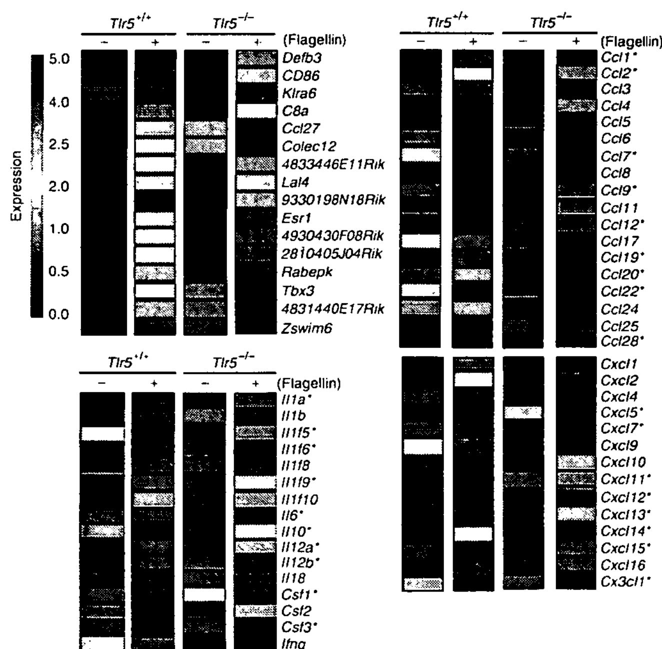

Figure 2 Gene expression induced by flagellin stimulation in IECs. Microarray analysis of IECs from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice stimulated with medium alone (–) or 1 μg/ml of flagellin (+). *, genes judged as being statistically undetectable at all time points. There is flagellin-induced expression 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.

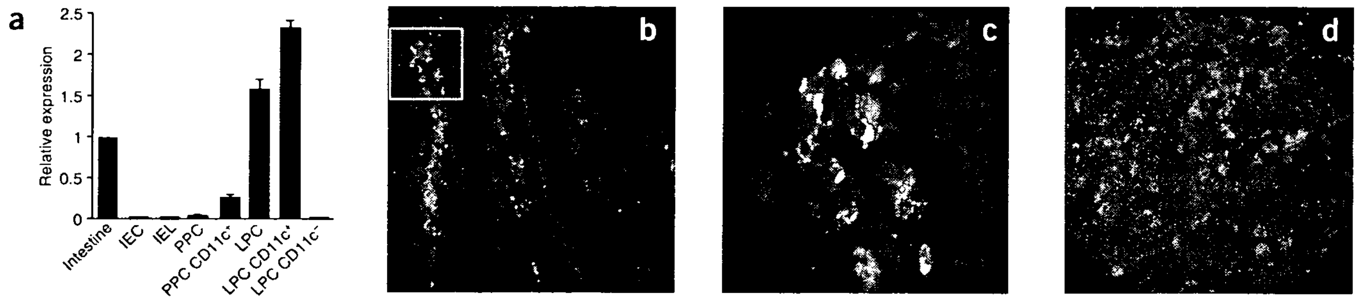

Figure 3 TLR5 is highly expressed on CD11c$^+$ LPCs. (a) Quantitative real-time PCR of *Tlr5* expression by the intestine (far left) and by various cell types of C57BL/6 mice. IEL, intestinal epithelial lymphocyte. Data are mean ± s.d. of triplicate samples from one representative of three independent 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.

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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,

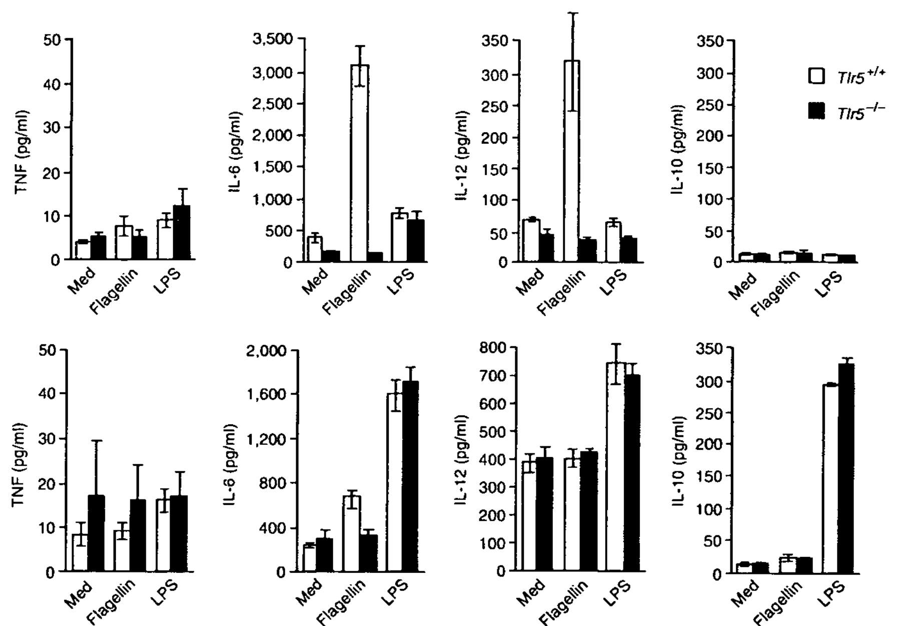

Figure 4 TLR5-mediated CD11c$^+$ LPC cytokine production. Enzyme-linked immunosorbent assay of cytokine production by CD11c$^+$ LPCs (top) and PPCs (bottom) from $Tlr5^{+/+}$ and $Tlr5^{-/-}$ mice. Cells were cultured with medium only, flagellin (1 μg/ml) or LPS (100 ng/ml). Data are mean ± s.d. of triplicate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

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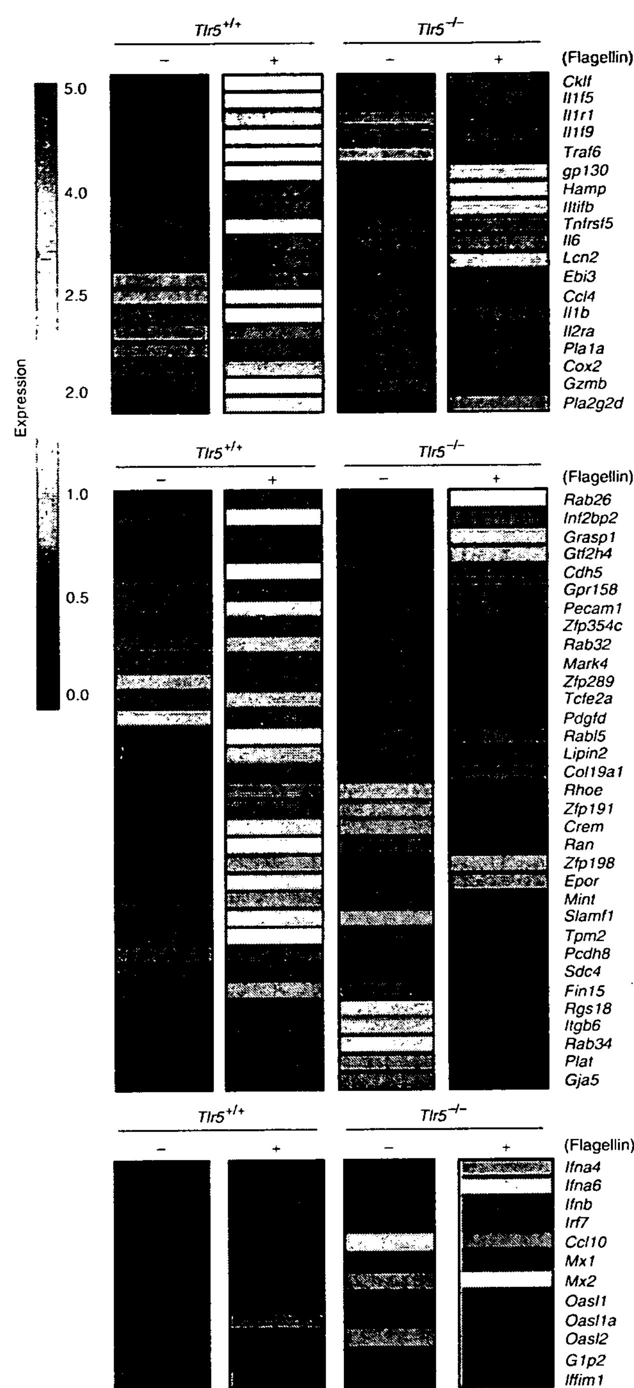

Figure 5 Flagellin-induced gene expression in CD11c$^+$ LPCs. Microarray analysis of CD11c$^+$ LPCs from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice left unstimulated (–) or stimulated with 1 μg/ml of flagellin (+). Upregulated genes encode cytokines (*Il6, Il1f5, Il1f9, Il1b, Ebi3* and *Iltifb*), cytokine 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

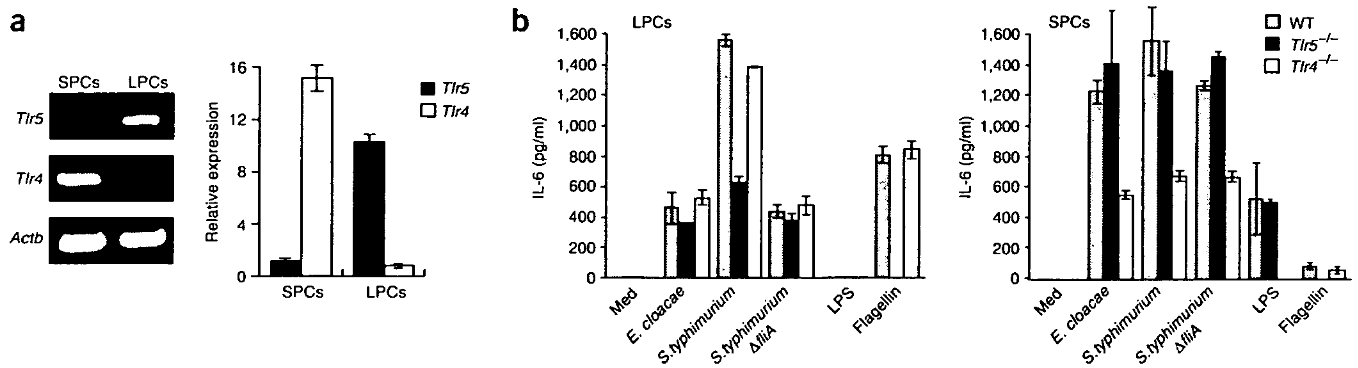

Figure 6 CD11c+ LPCs detect pathogenic bacteria via TLR5. (a) Quantitative real-time PCR of *Tlr5* and *Tlr4* expression in CD11c+ SPCs and CD11c+ LPCs of C57BL/6 mice. *Actb* encodes β-actin (loading control). Graphed data are mean ± s.d. of triplicate samples from one representative of three independent 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

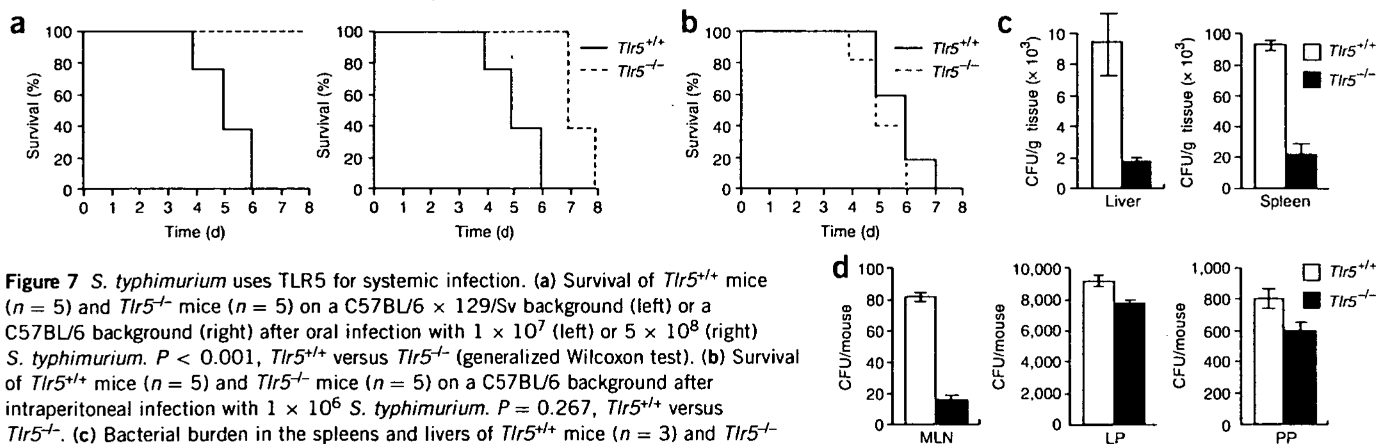

Figure 7 *S. typhimurium* uses TLR5 for systemic infection. (a) Survival of *Tlr5*$^{+/+}$ mice ($n = 5$) and *Tlr5*$^{-/-}$ mice ($n = 5$) on a C57BL/6 × 129/Sv background (left) or a C57BL/6 background (right) after oral infection with 1×10^7 (left) or 5×10^8 (right) *S. typhimurium*. $P < 0.001$, *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).

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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.

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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.

ACKNOWLEDGMENTS
We thank K. Smith and T. Hawn (Institute for Systems Biology, Seattle, Washington) for providing purified flagellin; C. Sasagawa and T. Suzuki (Institute of Medical Science, Tokyo, Japan) for providing bacteria; members of the DNA-chip Development Center for Infectious Diseases (RIMD, Osaka University, Osaka, Japan) for technical advice; N. Kitagaki for technical assistance; and M. Hashimoto for secretarial assistance. Supported by Special Coordination Funds, the Ministry of Education, Culture, Sports, Science and Technology, and Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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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TLR-Dependent Induction of IFN-β Mediates Host Defense against *Trypanosoma cruzi*[1]

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Host resistance to the intracellular protozoan parasite *Trypanosoma cruzi* depends on IFN-γ production by T cells and NK cells. However, the involvement of innate immunity in host resistance to *T. cruzi* remains unclear. In the present study, we investigated host defense against *T. cruzi* by focusing on innate immunity. Macrophages and dendritic cells (DCs) from MyD88$^{-/-}$TRIF$^{-/-}$ mice, in which TLR-dependent activation of innate immunity was abolished, were defective in the clearance of *T. cruzi* and showed impaired induction of IFN-β during *T. cruzi* infection. Neutralization of IFN-β in MyD88$^{-/-}$ macrophages led to enhanced *T. cruzi* growth. Cells from MyD88$^{-/-}$IFNAR1$^{-/-}$ mice also showed impaired *T. cruzi* clearance. Furthermore, both MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were highly susceptible to in vivo *T. cruzi* infection, highlighting the involvement of innate immune responses in *T. cruzi* infection. We further analyzed the molecular mechanisms for the IFN-β-mediated antitrypanosomal innate immune responses. MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages and DCs exhibited defective induction of the GTPase IFN-inducible p47 (IRG47) after *T. cruzi* infection. RNA interference-mediated reduction of IRG47 expression in MyD88$^{-/-}$ macrophages resulted in increased intracellular growth of *T. cruzi*. These findings suggest that TLR-dependent expression of IFN-β is involved in resistance to *T. cruzi* infection through the induction of IRG47. *The Journal of Immunology,* 2006, 177: 7059–7066.

The parasite *Trypanosoma cruzi* is an intracellular protozoan that causes Chagas' disease, a chronic systemic disorder affecting nearly 20 million people in Central and South America. Host defense against *T. cruzi* depends on a variety of cell populations, including NK, CD4$^+$ T cells, CD8$^+$ T cells, and Ig-producing B cells (1–3). In addition, macrophages and dendritic cells (DCs)[3] produce proinflammatory cytokines, such as IL-12, in response to invasion by *T. cruzi* (4–6). IL-12 induces IFN-γ production by NK, CD4$^+$ T cells, and CD8$^+$ T cells. In turn, IFN-γ induces NO production by macrophages and mediates the killing of *T. cruzi* (7, 8). This cytokine milieu is therefore responsible for host resistance to *T. cruzi* infection in vivo. However, it remains uncertain how innate immune cells, such as macrophages and DCs, mediate *T. cruzi*-induced immune responses during the early phase of infection. In addition, *T. cruzi* infection induces the production of type I IFNs (αβ IFN), which possess antiviral activities (9, 10). However, the nature of the involvement of type I IFNs in response to *T. cruzi* infection remains controversial (11).

A family of TLRs has been identified that recognize specific components of various microorganisms, including bacteria, viruses, fungi, and protozoan parasites (12). Recognition of microbial components by TLRs triggers the activation of innate immunity and the subsequent development of Ag-specific adaptive immunity. TLR-mediated signaling pathways originate from the cytoplasmic Toll/IL-1R (TIR) domains, which are conserved among all family members. A group of TIR domain-containing adaptors (MyD88, Toll/IL-1R domain-containing adaptor protein, TIR domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule) have been shown to be integral to these TLR signaling pathways (13). The TLR signaling pathways consist of two cascades: a MyD88-dependent pathway and a TRIF-dependent (MyD88-independent) pathway. The MyD88-dependent pathway mediates all TLR-induced productions of proinflammatory cytokines, including IL-12p40, whereas the TRIF-dependent pathway is indispensable for the induction of type I IFNs through TLR3 and TLR4.

Previous studies have analyzed the involvement of TLR-dependent activation of innate immunity in *T. cruzi* infection. TLR2, TLR4, and TLR9 have been implicated in the recognition of *T. cruzi*-derived components (6, 14–16), whereas mice lacking MyD88 were found to be susceptible to the acute phase of *T. cruzi* infection accompanied by defective proinflammatory cytokine production (17). However, even in MyD88-deficient mice, significant IFN-γ production was still observed, indicating the presence of MyD88-independent immune responses. Thus, the nature of the involvement of innate immunity in *T. cruzi* infection still remains to be precisely characterized.

In the present study, we analyzed the involvement of innate immune cells in *T. cruzi* infection using mice lacking both

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Received for publication May 31, 2006. Accepted for publication September 1, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology, as well as the Uehara Memorial Foundation, the Mitsubishi Foundation, the Takeda Science Foundation, the Tokyo Biochemical Research Foundation, the Kowa Life Science Foundation, the Osaka Foundation for Promotion of Clinical Immunology, and the Sankyo Foundation of Life Science.

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[3] Abbreviations used in this paper: DC, dendritic cell; TIR, Toll/IL-1R; TRIF, TIR domain-containing adaptor-inducing IFN-β; WT, wild type; siRNA, small interfering RNA; EF-1α, elongation factor-1α.

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0022-1767/06/$02.00

MyD88 and TRIF, in which all of the previously described TLR-mediated activation mechanisms of innate immunity are totally abolished.

Materials and Methods

Mice

$MyD88^{-/-}$ and $TRIF^{-/-}$ mice were generated as previously described (18, 19). Type I IFN receptor $(IFNAR1)^{-/-}$ mice were purchased from B & K Universal (20). Each mouse strain was backcrossed to C57BL/6 for at least five generations, and then used to generate double-mutant mice. $MyD88^{-/-}TRIF^{-/-}$ mice were generated by crossing $MyD88^{+/-}$ $TRIF^{+/-}$ mice. Littermate wild-type (WT) $(MyD88^{+/-}TRIF^{+/-})$, $MyD88^{-/-}$ $(MyD88^{-/-}TRIF^{+/-})$, and $TRIF^{-/-}$ $(MyD88^{+/-}TRIF^{-/-})$ mice were used for the experiments. $MyD88^{-/-}IFNAR1^{-/-}$ mice were generated by crossing $MyD88^{+/-}IFNAR1^{+/-}$ mice, and used for the experiments at 8–10 wk of age. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University.

Preparation of macrophages and DCs

To isolate peritoneal macrophages, mice were i.p. injected with 2 ml of 4% thioglycolate medium (Sigma-Aldrich), and peritoneal exudate cells were isolated from the peritoneal cavity at 3 days postinjection. The cells were incubated for 2 h and washed three times with HBSS. The remaining adherent cells were used as peritoneal macrophages in experiments. To prepare bone marrow-derived DCs or macrophages, bone marrow cells were prepared from the femur and tibia, passed through a nylon mesh and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 mM 2-ME, and 10 ng/ml GM-CSF (PeproTech) or 30% L cell culture supernatant. After 6 days, the cells were used as DCs or macrophages in experiments.

Parasites and experimental infection

The *T. cruzi* Tulahuén strain was maintained in vivo in IFN-$\gamma R^{-/-}$ mice by passages every other week (21). For in vitro experiments, macrophages or DCs (5×10^4) were infected with 5×10^4 trypomastigotes. After 6 h of infection, the cells were washed twice with PBS to remove the extracellular parasites and cultured in RPMI 1640 supplemented with 10% FBS for the indicated time periods. Trypomastigotes in the culture supernatants were counted microscopically. Alternatively, the cells were pulsed with 1 μCi of [^{3}H]uracil and cultured for 72 h. The cells were then harvested on glass fiber filters and the incorporated uracil was measured using a liquid scintillation counter. The net cpm was calculated by subtracting the background cpm in uninfected cultures from the cpm of the infected cultures. In some experiments, macrophages were infected with *T. cruzi* in the absence or presence of 10 ng/ml of an anti-IFN-β neutralizing Ab (YAMASA) for 6 h, washed and then further cultured with or without the anti-IFN-β Ab.

In other experiments, extracellular parasites were removed by repeated washing after 6h of infection, and the cells were incubated for a further 48 h. Subsequently, the cells were washed, fixed and stained using a Diff-Quik kit (Sysmex). The intracellular parasite numbers in 250 macrophages were counted under a light microscope. Counting was performed in a blinded manner by two independent investigators.

For in vivo experiments, mice were i.p. injected with plasma containing 2×10^3 or 1×10^4 trypomastigotes as indicated. The number of parasites in the blood of each animal was then counted microscopically using 5 μl of blood taken from the tail. Statistical significance was determined using a paired Student's t test. Differences were considered to be statistically significant at $p < 0.05$.

Measurement of cytokine production

Peritoneal macrophages or DCs (5×10^4) were infected with 5×10^4 *T. cruzi* for 6 h, extensively washed and cultured for 24 h. The culture supernatants were collected and analyzed for their levels of TNF-α by ELISA (Genzyme Techne) and NO using the Griess reagent (Dojindo Laboratories).

Quantitative real-time RT-PCR

Total RNA was isolated with an RNeasy mini kit (Qiagen), and 2 μg of the RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega) and oligo(dT) primers (Toyobo) after treatment with RQ1 DNase I (Promega). Quantitative real-time PCR was performed in an ABI 7000 (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). All data were normalized to the corresponding level of elongation factor-1α (EF-1α) expression, and the fold difference relative to the EF-1α level was calculated. The amplification conditions were: 50°C (2 min), 95°C (10 min), and 40 cycles of 95°C (15 s), and 60°C (60 s). Each experiment was performed independently at least three times, and the results of one representative experiment are shown. All primers were purchased from Assay on Demand (Applied Biosystems).

RNA interference

For small interfering RNA (siRNA) experiments, dsRNA duplexes targeting the coding region of the GTPase IFN-inducible p47 (IRG47) (5'-GGUGGAUAGUGACUUAUAUtt-3') were synthesized by Ambion. Bone marrow cells were cultured in the presence of 30% L cell culture supernatant for 6 days. The differentiated bone marrow macrophages were then harvested by 5 mM EDTA treatment and transfected with 1.5 μg of the siRNA using Nucleofector and a Mouse Macrophage Nucleofector kit (Amaxa Biosystems) according to the manufacturer's instructions. The cells were immediately transferred to culture medium and cultured for 18 h. Next, cells were infected with *T. cruzi* for 48 h, and parasite growth was analyzed. To determine the efficiency of gene silencing, cells were infected with *T. cruzi* for 6 h, and the expression of IRG47 mRNA was analyzed by quantitative real-time RT-PCR.

Results

Increased growth of T. cruzi *in $MyD88^{-/-}TRIF^{-/-}$ DCs and macrophages*

To study the direct involvement of innate immunity in *T. cruzi* infection, bone marrow-derived DCs prepared from WT, $MyD88^{-/-}$, $TRIF^{-/-}$, or $MyD88^{-/-}TRIF^{-/-}$ mice were infected with *T. cruzi*. After 6 h of *T. cruzi* infection, the cells were extensively washed and changed to fresh medium. After culture periods of 4, 5, and 7 days, the number of trypomastigotes released into the culture supernatants were counted (Fig. 1*A*). The culture supernatant of $TRIF^{-/-}$ DCs contained a similar number of trypomastigotes to that of WT DCs. For $MyD88^{-/-}$ DCs, the number of trypomastigotes increased after 5 and 7 days of infection. Furthermore, for $MyD88^{-/-}TRIF^{-/-}$ DCs, the number of trypomastigotes increased considerably. Next, peritoneal macrophages were infected with *T. cruzi* (Fig. 1*B*). The number of trypomastigotes in the culture supernatant of $MyD88^{-/-}$ macrophages was slightly increased compared with those of WT or $TRIF^{-/-}$ cells after 5 and 7 days of infection. For $MyD88^{-/-}TRIF^{-/-}$ macrophages, larger numbers of trypomastigotes were observed compared with the other cell genotypes after 7 days of infection. Next, replication of *T. cruzi* within macrophages was assessed based on [^{3}H]uracil incorporation (Fig. 1*C*). Intracellular growth of *T. cruzi* was slightly increased in $MyD88^{-/-}$ macrophages, and markedly increased in $MyD88^{-/-}TRIF^{-/-}$ cells compared with WT cells. Bone marrow-derived macrophages were also infected with *T. cruzi* and cultured for 48 h, before the number of intracellular parasites was counted. The number of infected cells did not differ among the genotypes (data not shown). However, infected $MyD88^{-/-}TRIF^{-/-}$ macrophages contained an increased number of parasites after 48 h of infection (Fig. 1, *D* and *E*). Thus $MyD88^{-/-}$ DCs and macrophages showed a slight increase in *T. cruzi* growth, whereas $MyD88^{-/-}TRIF^{-/-}$ cells showed a marked increase in growth, indicating that $MyD88^{-/-}TRIF^{-/-}$ DCs and macrophages were defective in the clearance of *T. cruzi*.

Defective T. cruzi *induction of proinflammatory mediators in $MyD88^{-/-}$ macrophages and DCs*

The killing of parasites by macrophages has been shown to be mediated by TNF-α and NO (22–25). Therefore, we next analyzed the production of TNF-α and NO by *T. cruzi*-infected peritoneal macrophages (Fig. 2). Both WT and $TRIF^{-/-}$ macrophages secreted TNF-α and NO in response to *T. cruzi* infection. In contrast,

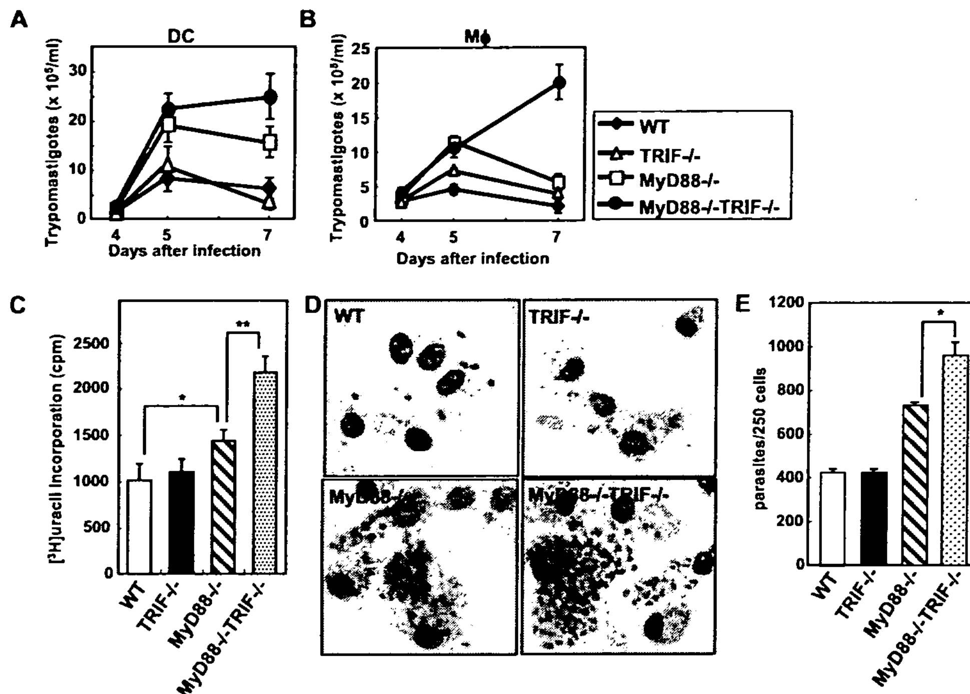

FIGURE 1. Defective *T. cruzi* clearance in MyD88$^{-/-}$TRIF$^{-/-}$ DCs and macrophages. Bone marrow-derived DCs (*A*) or peritoneal macrophages (Mϕ) (*B*) from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$TRIF$^{-/-}$ mice were seeded onto 96-well plates, and infected with *T. cruzi* for 6 h. The cells were then washed to remove the extracellular parasites and cultured for the indicated periods, before the numbers of trypomastigotes in the culture supernatants were counted. Data are representative of four independent experiments. *C*, Peritoneal macrophages were infected with *T. cruzi*, washed and cultured in the presence of [^{3}H]uracil for 72 h, before the [^{3}H]uracil incorporation was measured. *, $p < 0.01$; **, $p < 0.005$. *D* and *E*, Bone marrow-derived macrophages 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. The intracellular parasites were counted, and the data represent the mean + SD of the number of parasites per 250 macrophages. *, $p < 0.02$.

secretion of these mediators was severely reduced in both MyD88$^{-/-}$ and MyD88$^{-/-}$TRIF$^{-/-}$ macrophages, and no significant differences were observed between the two genotypes. These findings indicate that *T. cruzi*-induced production of TNF-α and NO was dependent on MyD88, but that the higher susceptibility to *T. cruzi* infection of MyD88$^{-/-}$TRIF$^{-/-}$ macrophages was not due to defective induction of these mediators.

Defective T. cruzi *induction of IFN-inducible genes in MyD88$^{-/-}$TRIF$^{-/-}$ macrophages and DCs*

Next, we tried to identify which genes were selectively less active in *T. cruzi*-infected MyD88$^{-/-}$TRIF$^{-/-}$ DCs. *T. cruzi* infection has been shown to induce IFN-β (9, 10). Therefore, we analyzed *T. cruzi*-induced gene expression focusing on IFN-β and IFN-inducible chemokines as well as proinflammatory cytokines in peritoneal macrophages and DCs from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, and MyD88$^{-/-}$TRIF$^{-/-}$ mice by quantitative real-time RT-PCR. In WT and TRIF$^{-/-}$ macrophages, *T. cruzi* infection led to robust induction of TNF-α and IL-12p40 mRNAs (Fig. 3*A*). In contrast, both MyD88$^{-/-}$ and MyD88$^{-/-}$TRIF$^{-/-}$ macrophages showed defective induction of TNF-α and IL-12p40. Expression of the mRNAs for IFN-β and IFN-inducible genes, such as *Ccl2* (MCP-1), *Ccl5* (RANTES), and *Cxcl10* (IP-10) was induced in *T. cruzi*-infected WT DCs (Fig. 3*B*). In contrast, *T. cruzi*-induced expression of IFN-α4 mRNA was not observed in any of the macrophage and DC genotypes (data not shown). In MyD88$^{-/-}$ DCs, *T. cruzi*-induced expression of *Ccl2*, *Ccl5*, and *Cxcl10* was only slightly reduced. However, DCs from TRIF$^{-/-}$ and MyD88$^{-/-}$TRIF$^{-/-}$ mice showed severely impaired induction of these genes after *T. cruzi* infection. Peritoneal macrophages from each genotype showed similar patterns of *T. cruzi*-induced gene expression (Fig. 3*C*). Thus, MyD88$^{-/-}$ macrophages and DCs showed defective induction of proinflammatory cytokine genes during *T. cruzi* infection, whereas TRIF$^{-/-}$ cells showed defective induction of IFN-β and IFN-inducible genes during the infection. Furthermore, MyD88$^{-/-}$TRIF$^{-/-}$ cells displayed defective expression of all these genes.

IFN-β-mediated inhibition of T. cruzi *growth in MyD88$^{-/-}$ macrophages*

MyD88$^{-/-}$TRIF$^{-/-}$ macrophages and DCs displayed defective clearance of *T. cruzi* with impaired expression of IFN-β and IFN-inducible genes. Therefore, we next addressed whether IFN-β is involved in the resistance to *T. cruzi* infection in MyD88$^{-/-}$ macrophages. Peritoneal macrophages from WT and MyD88$^{-/-}$ mice were infected with *T. cruzi* in the presence of an anti-IFN-β neutralizing Ab, and intracellular growth of *T. cruzi* was measured (Fig. 4). In WT macrophages, *T. cruzi* growth remained unaltered by the addition of the anti-IFN-β Ab. In contrast, anti-IFN-β Ab addition dramatically increased the intracellular growth of *T. cruzi* in MyD88$^{-/-}$ macrophages. These findings indicate the possible involvement of IFN-β in resistance to *T. cruzi* infection in the absence of MyD88.

High-sensitivity to T. cruzi *infection in MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages*

To further address whether IFN-β is involved in the resistance to *T. cruzi* infection, we generated mice lacking both MyD88 and the IFNAR1 subunit of the $\alpha\beta$ IFN receptor complex (MyD88$^{-/-}$IFNAR1$^{-/-}$ mice). Bone marrow-derived macrophages were infected with *T. cruzi*, washed, and cultured. After culture periods of 4, 5, and 7 days, the numbers of trypomastigotes in the culture

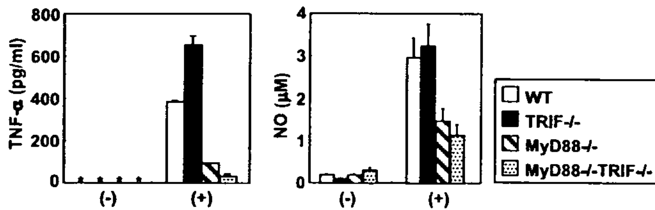

FIGURE 2. Defective production of TNF-α and NO in *T. cruzi*-infected MyD88$^{-/-}$ macrophages. Peritoneal macrophages from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$TRIF$^{-/-}$ mice were infected with (+) or without (−) *T. cruzi* for 6 h, washed to remove the extracellular parasites, and cultured for 24 h. The levels of TNF-α and NO in the culture supernatants were measured. *, Not detected.

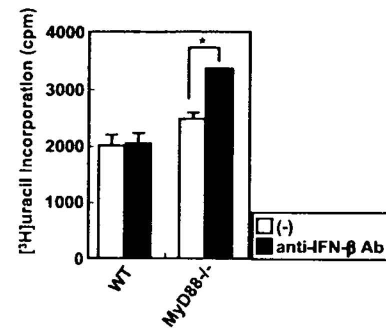

FIGURE 4. Effect of an anti-IFN-β neutralizing Ab on *T. cruzi* growth in macrophages. Peritoneal macrophages from WT or MyD88$^{-/-}$ mice were infected with *T. cruzi* for 6 h in the presence or absence of an anti-IFN-β neutralizing Ab, washed, and cultured in the presence of [^{3}H]uracil for 72 h. The [^{3}H]uracil incorporation was then measured. *, $p < 0.005$.

supernatants were counted (Fig. 5*A*). As mentioned, the culture supernatant of MyD88$^{-/-}$ macrophages contained a larger number of trypomastigotes than that of WT macrophages. In the supernatant of IFNAR1$^{-/-}$ macrophages, a slight increase in the number of trypomastigotes was observed compared with WT cells. Furthermore, the number of trypomastigotes in the culture supernatant of MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages was considerably increased. Next, intracellular replication of *T. cruzi* was assessed by counting [^{3}H]uracil incorporation (Fig. 5*B*). MyD88$^{-/-}$ and IFNAR1$^{-/-}$ macrophages showed slightly increased growth rates of *T. cruzi*. However, MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages showed markedly increased growth rates of *T. cruzi* compared with MyD88$^{-/-}$ or IFNAR1$^{-/-}$ cells. Furthermore, at 48 h after the *T. cruzi* infection, increased numbers of parasites were observed in MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages (Fig. 5, *C* and *D*). Thus, IFNAR1$^{-/-}$ macrophages displayed a slightly increased sensitivity to *T. cruzi* infection, whereas MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages displayed a higher sensitivity to the infection. These findings suggest that IFN-β is responsible for resistance to *T. cruzi* infection and that this responsibility becomes evident in the absence of MyD88.

High-sensitivity MyD88$^{-/-}$IFNAR1$^{-/-}$ mice to T. cruzi *infection*

Macrophages are the primary site of *T. cruzi* replication, and thus act as the major cell population for controlling the infection in vivo, especially for reticulotropic strains such as the Tulahuén strain used in the present study (21, 26). Therefore, we next addressed whether IFN-β mediates antitrypanosomal responses in vivo. Mice were i.p. infected with *T. cruzi*, and the parasitemia was monitored (Fig. 6*A*). In WT and TRIF$^{-/-}$ mice, the trypomastigote counts in the sera peaked by day 13 of the infection, and subsequently decreased. In IFNAR1$^{-/-}$ mice, serum trypomastigotes were slightly increased compared with WT or TRIF$^{-/-}$ mice, and peaked around 11–13 days of infection. In MyD88$^{-/-}$ mice, the parasite counts were increased at 13 days of infection. In MyD88$^{-/-}$TRIF$^{-/-}$ mice, the serum parasite counts continued to increase, and these mice showed much higher levels of parasitemia by day 15 of infection than levels found in MyD88$^{-/-}$ mice. In MyD88$^{-/-}$IFNAR1$^{-/-}$ mice, the parasite counts increased in a similar manner

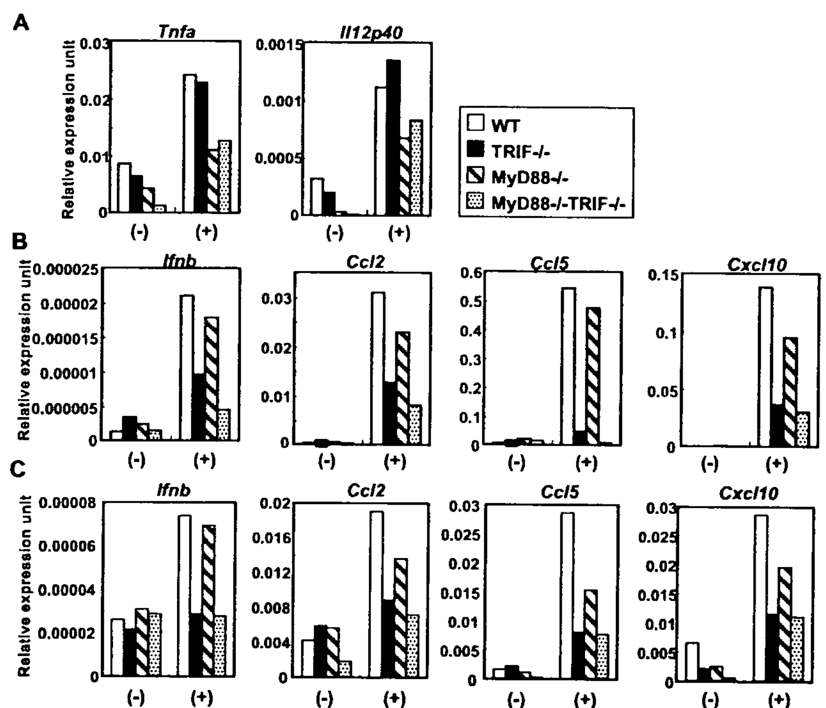

FIGURE 3. *T. cruzi*-induced expression of inflammatory genes in macrophages and DCs. *A*, Peritoneal macrophages from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$TRIF$^{-/-}$ mice were cultured in the presence (+) or absence (−) of *T. cruzi* for 6 h. Total RNA was then extracted and analyzed for the expressions of *Tnfa* or *Il-12p40* by quantitative real-time RT-PCR. The data are shown as the relative mRNA levels normalized by the corresponding EF-1α mRNA level. Bone marrow-derived DCs (*B*) or peritoneal macrophages (*C*) from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$TRIF$^{-/-}$ mice were cultured in the presence (+) or absence (−) of *T. cruzi* for 6 h. Total RNA was then extracted and analyzed for the expressions of *Ifnb*, *Ccl2*, *Ccl5*, and *Cxcl10* by quantitative real-time RT-PCR. Data are presented in relative expression units and have been normalized to the corresponding EF-1α mRNA level.

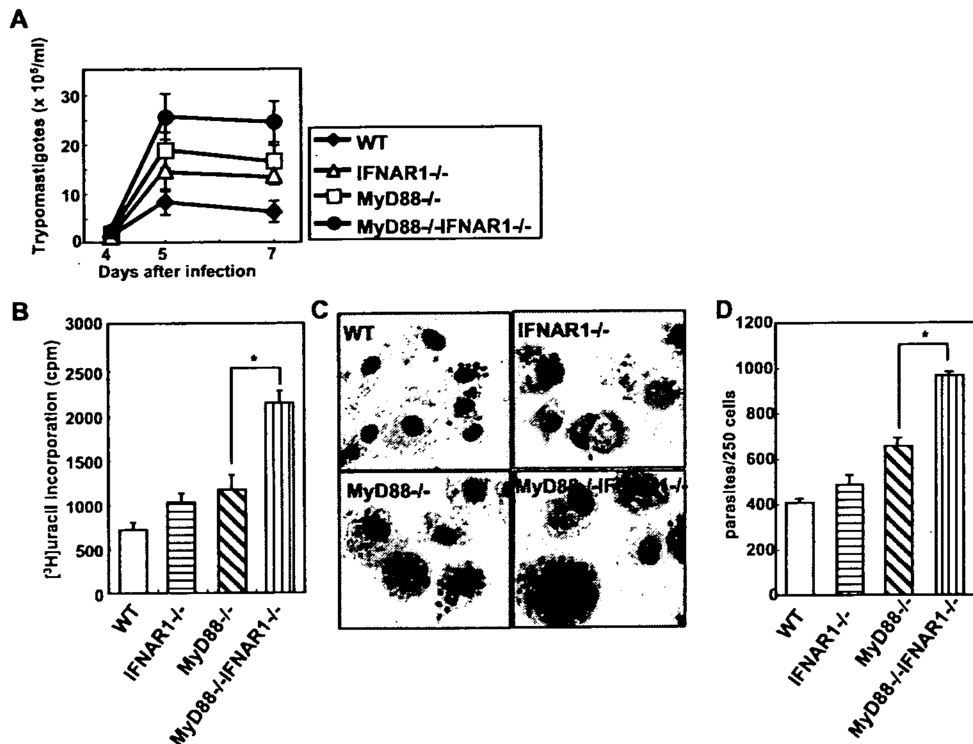

FIGURE 5. Increased *T. cruzi* growth in MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages. *A*, Bone marrow-derived macrophages from WT, MyD88$^{-/-}$, IFNAR1$^{-/-}$, or MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were infected with *T. cruzi* for 6 h, washed to remove the extracellular parasites, and cultured for the indicated periods. The trypomastigotes in the culture supernatants were counted. Data are representative of four independent experiments. *B*, Peritoneal macrophages were infected with *T. cruzi*, washed, and cultured in the presence of [^{3}H]uracil for 72 h. The [^{3}H]uracil incorporation was then measured. *, $p < 0.0001$. *C* and *D*, Bone marrow-derived macrophages from each genotype 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. *, $p < 0.02$.

to those in MyD88$^{-/-}$TRIF$^{-/-}$ mice. We further monitored the mortality of the mice after *T. cruzi* infection (Fig. 6*B*). WT, TRIF$^{-/-}$, and IFNAR1$^{-/-}$ mice were resistant to *T. cruzi* infection, and all the mice survived for more than 19 days after the infection, whereas MyD88$^{-/-}$ mice started to die around 15 days after the infection, and about half of the mice had died within 19 days. In contrast, all the MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ mice died within 19 days of the infection. Thus, MyD88$^{-/-}$TRIF$^{-/-}$ and MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were more sensitive to in vivo *T. cruzi* infection than MyD88$^{-/-}$ mice, suggesting that IFN-β mediates in vivo resistance to *T. cruzi* infection, and this effect becomes evident in the absence of MyD88.

Involvement of IFN-β-inducible IRG47 in resistance to T. cruzi *infection*

Next, we addressed the molecular mechanisms of the IFN-β-mediated resistance to *T. cruzi* infection in innate immune cells. The family of p47 GTPases has been shown to control innate immune responses to intracellular pathogens, including protozoan parasites (27, 28). In addition, expression of p47 GTPases, such as LRG47

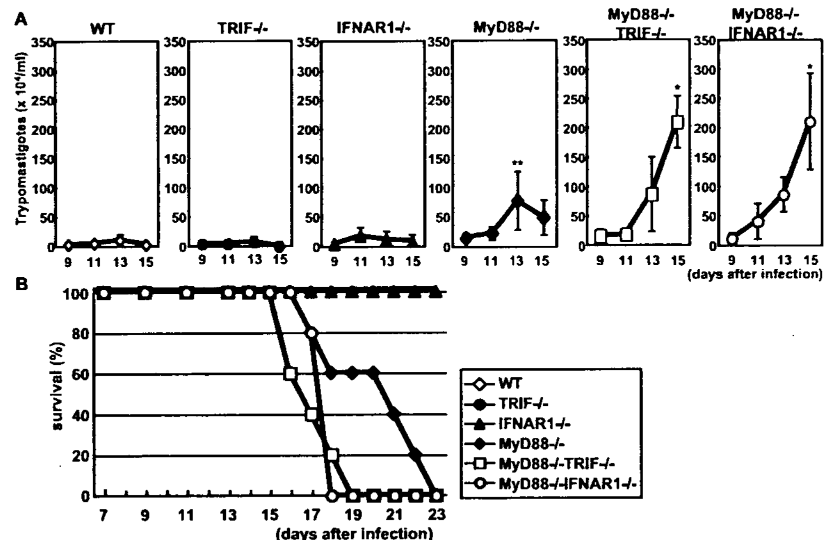

FIGURE 6. High-sensitivity MyD88$^{-/-}$IFNAR1$^{-/-}$ mice to *T. cruzi* infection. WT (n = 9), TRIF$^{-/-}$ (n = 10), IFNAR1$^{-/-}$ (n = 10), MyD88$^{-/-}$ (n = 5), MyD88$^{-/-}$TRIF$^{-/-}$ (n = 5), and MyD88$^{-/-}$IFNAR1$^{-/-}$ (n = 5) mice were i.p. infected with 1 × 10^4 *T. cruzi*. Parasitemia (*A*) and mortality (*B*) were monitored at the indicated times after infection. *, $p < 0.001$ compared with MyD88$^{-/-}$ mice and **, $p < 0.005$ compared with control mice.

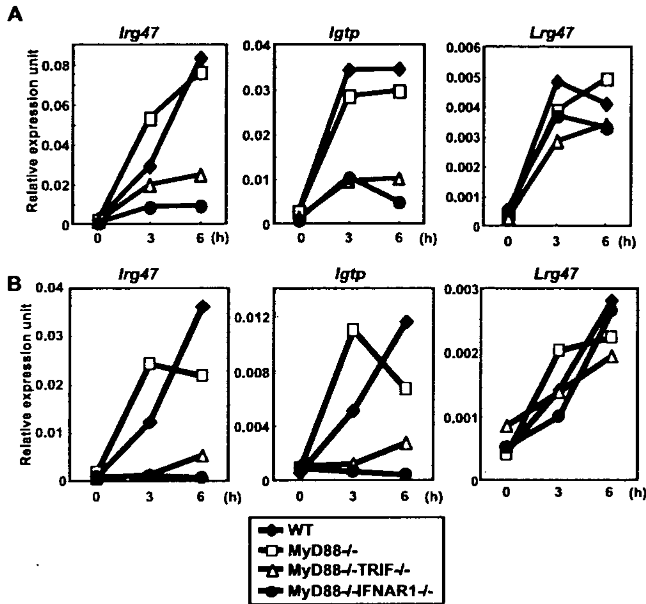

FIGURE 7. Impaired expression of IRG47 in *T. cruzi*-infected MyD88$^{-/-}$TRIF$^{-/-}$ mice. Bone marrow-derived macrophages (*A*) or DCs (*B*) from WT, MyD88$^{-/-}$, MyD88$^{-/-}$TRIF$^{-/-}$, or MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were infected with *T. cruzi* for 3 or 6 h. Next, total RNA was extracted 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

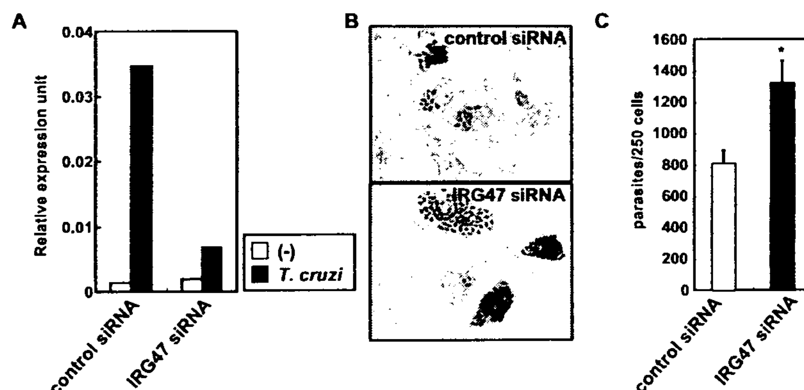

FIGURE 8. IRG47 mediates antitrypanosomal activity in MyD88$^{-/-}$ mice. *A*, Bone marrow-derived macrophages were transfected with IRG47 or control siRNA and cultured for 18 h. The cells were then infected with *T. cruzi* for 6 h and analyzed for the expression of IRG47 mRNA by quantitative real-time 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.

Acknowledgments

We thank Y. Yamada and K. Takeda for technical assistance, and M. Kurata for secretarial assistance.

Disclosures

The authors have no financial conflict of interest.

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結核菌薬剤感受性検査のための BACTEC MGIT 960 AST の評価：外部精度管理菌株を用いた研究

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要旨：〔目的〕迅速な結核菌薬剤感受性検査法であるBACTEC MGIT 960結核菌薬剤感受性検査用ミジットシリーズ（Mycobacterium Growth Indicator Tube Antimicrobial Susceptibility Testing: MGIT AST）の精度を調べた。〔材料と方法〕評価にはWHO/IUATLDが薬剤感受性検査の外部精度アセスメントに使用している菌株と結核菌H37Rvを用いた。MGIT ASTとMiddlebrook 7H10寒天培地による比率法（比率法）でイソニアジド（INH），リファンピシン（RFP），ストレプトマイシン（SM），エタンブトール（EB）に対する感受性を測定した。得られた結果はWHO/IUATLDのSupranational Reference Laboratory Network（SRLN）の結果と比較した。〔結果〕INHとRFPの検査では，SRLNの成績を標準としたときMGIT ASTの感度，特異性，一致率，再現性のいずれも100%であった。SMの検査について，MGIT ASTのSRLNの結果との一致率は97.9%，EBの検査は91.5%であり，主要4薬剤について90%以上の一致率を示した。〔結論〕今回の結果からMGIT ASTはWHO/IUATLDが目標として掲げた薬剤感受性検査の精度，すなわち検査の感度，特異性，再現性および一致率を満足させる迅速な検査であると考えられる。

キーワーズ：MGIT AST，薬剤感受性検査，結核菌，WHO/IUATLD

はじめに

薬剤感受性検査の結果は患者の治療のみならず薬剤耐性結核菌の出現にも影響を与えることから，検査の精度管理は重要である。薬剤感受性検査は抗酸菌の検査の中で最も精度管理の難しい検査である。世界保健機関（World Health Organization: WHO）と国際結核肺疾患予防連合（International Union Against Tuberculosis and Lung Disease: IUATLD）は世界的規模で薬剤耐性結核のサーベイランスを実施するにあたり，世界で20数カ所の研究所または大学の研究室をSupranational Reference Laboratory（SRL）に選び，薬剤感受性検査の精度管理研究をスタートさせた[1]。WHO/IUATLDのコーディネーターからSRLに薬剤耐性菌を含む結核菌を送付し，SRLのネットワーク（SRLN）で標準化し，それらの菌株をサーベイランス実施国の精度管理に用いている。わが国でも日本結核病学会抗酸菌検査法検討委員会が上記の菌株を用いて医療機関や検査センターを対象に薬剤感受性検査の外部精度アセスメントを実施しており[2)3)]，その結果手技の習熟や使用する方法による特性を把握することなど，改めて精度管理の重要性が明らかになった。

検査の所要日数の長短は患者の治療や管理に影響することから，時宜にかなった検査結果の報告は微生物検査室に課せられた重要な責務である。わが国で現在用いている小川培地による結核菌の薬剤感受性検査は結果を得るまでに約1カ月を要することから，より短時間で結果が得られる検査が望まれている。米国のCenters for Disease Control and Prevention（CDC）から出された検査の所要日数についての提案では，検体入手後結核菌の薬剤感受性検査の結果を30日以内に臨床医に報告することとしており[4)5)]，これは固形培地を用いた従来法で達成することは不可能である。液体培地を用いるBACTEC 460 TBやMGIT ASTシステムの迅速性と有用性に関する報告は数多くみられる[6)~12)]。米国やヨーロッパ諸国では液

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(Received 19 Oct. 2005/Accepted 9 Nov. 2005)

体培地を用いる薬剤感受性検査が推奨されており，わが国でも今後普及していくものと思われる。

以前にわれわれは臨床分離株を用いMGIT ASTと小川法の成績を比較した結果を報告した[10]。今回WHO/IUATLDが薬剤感受性検査の外部精度アセスメントに使用している菌株を用い，MGIT ASTの精度を再評価したので報告する。

検査材料と方法

（1）菌株

WHO/IUATLDのコーディネーターから分与された結核菌50株と結核菌H37Rv (ATCC 27294) を評価に用いた。これらの結核菌はWHO/IUATLDのSRLNで標準化された菌株である。

（2）接種菌液の調製

被検菌を2%小川培地に接種後37℃で約4週間培養した。培地表面の集落を満遍なくかきとり5 mLのMiddlebrook 7H9培地に懸濁後37℃で静置培養した。毎日1回撹拌し，培養液の濁度がMcFarland #0.5を超えるまで培養した。濁度計と滅菌蒸留水を用い，培養液からMcFarland #0.5濁度の菌液を調整した。

（3）MGIT AST

BACTEC MGIT 960結核菌薬剤感受性検査用ミジットシリーズの添付文書に従い，結核菌薬剤感受性用MGITチューブに専用サプリメントと薬剤を添加した。検査に用いた薬剤の濃度は，INH: 0.1 μg/mL，RFP: 1.0 μg/mL，SM: 1.0 μg/mL，EB: 5.0 μg/mLであった。McFarland #0.5に調整した菌液を滅菌蒸留水で5倍希釈し，薬剤添加MGITチューブへの接種菌液とした。コントロール用MGITチューブへの接種には，接種菌液を滅菌蒸留水でさらに100倍希釈したものを用いた。菌液を各MGITチューブに0.5 mL接種後，直ちにBACTEC MGIT 960全自動抗酸菌培養装置（MGIT 960）で培養を開始した。MGIT ASTは，培養開始4日目より13日目までの間にコントロール用MGITチューブの菌発育を示す蛍光強度が一定値を越えた時点で薬剤添加MGITチューブの蛍光強度を測定し，個々の薬剤に対する被検菌の感受性を判定するシステムである。

（4）Middlebrook 7H10培地による比率法

National Committee for Clinical Laboratory Standards（現CLSI: Clinical and Laboratory Standards Institute）M24-Aに記載されている方法に従いMiddlebrook 7H10寒天培地を調製した[13]。検査に用いた薬剤の濃度は，INH: 0.2 μg/mL，RFP: 1.0 μg/mL，SM: 2.0 μg/mL，EB: 5.0 μg/mLであった。McFarland #0.5に調整した菌液を滅菌蒸留水で100倍希釈し，薬剤添加Middlebrook 7H10寒天培地への接種菌液とした。コントロール用Middlebrook 7H10寒天培地への接種には，接種菌液を滅菌蒸留水でさらに100倍希釈したものを用いた。菌液を各培地に塗布後5%CO_2条件下37℃で培養し，3週目に判定した。

（5）評価方法

MGIT ASTおよびMiddlebrook 7H10寒天培地による比率法（比率法）の結果について，WHO/IUATLDのSRLNの結果を標準として感度，特異性，一致率，耐性的中率 (PV-R)，感受性的中率（PV-S）を計算し評価した[1]。感度はSRLNの結果が耐性のものを正しく耐性と判定した割合，特異性はSRLNの結果が感受性のものを正しく感受性と判定した割合，一致率はSRLの結果がそれぞれの薬剤について耐性，もしくは感受性と判定したものを同様に判定した割合である。PV-Rは耐性と判定したとき，その判定が正解である確率，PV-Sは感受性と判定したときの正解率である。なお，今回評価に用いた菌株の中でSRLN内の一致率が70%以下の菌株はRFPの検査で6株，SMで3株，EBで3株あり，これらはそれぞれの薬剤についての精度計算から除外した。

MGIT ASTの再現性は，被検菌の中から無作為に選んだ6菌株を用い，MGIT ASTの4薬剤について1日3回，3日間繰り返し（1菌株，1薬剤につき計9回の検査）測定し，SRLNの結果との一致率で評価した。

結　果

（1）MGIT ASTの再現性試験

無作為に選んだ6株を用いMGIT ASTシステムの再現性を調べた。計212検査を行い，全体の一致率は95.3%であった（Table 1)。INHとRFPの試験の一致率は100%であったが，SMとEBの試験でSRLNの結果と一部異なる成績がみられた。特にEBの試験で一致率は90%以下であった。

（2）外部精度アセスメント株を用いたMGIT ASTシステムの評価

WHO/IUATLDのSRLNで精度アセスメントに使用している50株の臨床分離結核菌および結核菌標準株H37Rvを用いMGIT ASTの精度を評価した。同時にCLSIが標準法としているMiddlebrook 7H10寒天培地を用いる比率法で検査を行い比較した。

INH感受性検査では，MGIT ASTの結果はSRLNの結果とすべて一致した（Table 2)。感度，特異性，一致率，PV-R，PV-Sのいずれも100%であった（Table 3)。比率法の結果はMGIT ASTの成績と同様にSRLNの結果といずれも100%一致した。

使用した50株のうち，6株はRFPの感受性検査でSRLN内の一致率が70%以下であったためRFPの精度計算から除外し，44株の結果で評価した。RFP感受性検査ではINHの検査と同様にMGIT ASTの結果はSRLNの

Table 1 Reproducibility testing of the BACTEC MGIT 960 AST system

Drug	No. of results	No. of results agreeing with SRLN	Agreement (%)
Isoniazid	53	53	100
Rifampin	53	53	100
Streptomycin	53	49	92.5
Ethambutol	53	47	88.7
Total	212	202	95.3

Reproducibility was assessed with six strains of *M. tuberculosis* in triplicate from three separately prepared inocula (i.e., nine replicate per strain).

Table 2 Comparison of drug susceptibility test results

Drug		No. of isolates with the following results				
	SRLN	R	R	R	S	S
	MGIT	R	R	S	S	S
	7H10	R	S	R	R	S
Isoniazid		32	0	0	0	18
Rifampin		18	0	0	0	26
Streptomycin		20	1	1	0	25
Ethambutol		15	0	4	2	26

SRLN: Referee results of the WHO/IUATLD Supranational Reference Laboratory Network
MGIT: BACTEC MGIT 960 AST system
7H10: Proportion method on Middlebrook 7H10 agar

Table 3 Comparison of the results with the BACTEC MGIT 960 AST system or proportion method on Middlebrook 7H10 agar with the results of the WHO SRL Network

	Sensitivity	Specificity	PV-R	PV-S	Agreement
BACTEC MGIT AST					
Isoniazid	100	100	100	100	100
Rifampin	100	100	100	100	100
Streptomycin	95.5	100	100	96.2	97.9
Ethambutol	78.9	100	100	87.5	91.5
Proportion method					
Isoniazid	100	100	100	100	100
Rifampin	100	100	100	100	100
Streptomycin	95.5	100	100	96.2	97.9
Ethambutol	100	92.9	90.5	100	95.7

Sensitivity: Ability to detect true resistance
Specificity: Ability to detect true susceptibility
PV-R: Predictive value for resistance
PV-S: Predictive value for susceptibility

結果と完全に一致した。感度，特異性，一致率，PV-R，PV-Sはすべて100％であった。比率法の結果もMGIT ASTとまったく同様であり，いずれも100％であった。

SMに対する感受性検査でSRLNの結果と不一致となった菌株はMGIT ASTと比率法でそれぞれ1株ずつ認められた。それらのうち1株はSRLNの結果が耐性をMGIT ASTで感受性として，別の株はSRLNで耐性と判定した株を比率法で感受性と判定していた（Table 2）。Table 3に示したようにSM検査におけるMGIT ASTおよび比率法のSRLNの結果との一致率は97.9％であった。

EBに対する感受性検査でSRLNの成績と不一致の結果を示した菌株はMGIT ASTで4株，比率法で2株認められた。MGIT ASTで不一致であった4株はいずれもSRLNで耐性と判定したものを感受性と判定していた。一方比率法で不一致の結果を示した2株ともSRLNで感受性と判定した菌株であり比率法で耐性と判定していた。MGIT ASTのSRLNの結果との一致率は91.5％，比率法の一致率は95.7％であった。

MGIT AST検査用チューブに菌接種後感受性の検査結果が得られるまでに要した日数は6日～13日の範囲であり，中央値は7日であった。一方比率法の所要日数は3週間であった。