表2 WHO 地域別の結核の現状（2004 年 WHO 推定）

| WHO 地域 | アフリカ | 南北アメリカ | 東地中海 | ヨーロッパ | 東南アジア | 西太平洋 | 総数 |
|---|---|---|---|---|---|---|---|
| 人口（100 万人） | 722 | 880 | 530 | 881 | 1,633 | 1,740 | 6,387 |
| 全結核 | | | | | | | |
| 人数（千人） | 2,573 | 363 | 645 | 445 | 2,967 | 1,925 | 8,918 |
| 率（人口 10 万人対） | 356 | 41 | 122 | 50 | 182 | 111 | 140 |
| HIV 感染率（%） | 33 | 10.2 | 2.4 | 4.7 | 3.9 | 1.4 | 13 |
| 塗抹陽性 | | | | | | | |
| 人数（千人） | 1,098 | 161 | 289 | 199 | 1,327 | 865 | 3,939 |
| 率（人口 10 万人対） | 224 | 22 | 96 | 31 | 113 | 95 | 95 |
| HIV 感染率（%） | 6.5 | 2.0 | 0.3 | 0.9 | 0.7 | 0.2 | 2.1 |
| 全結核死亡 | | | | | | | |
| 死亡数（千人） | 587 | 52 | 142 | 69 | 535 | 307 | 1,693 |
| 結核死亡率（人口 10 万人対） | 81 | 5.9 | 27 | 7.8 | 33 | 18 | 27 |
| HIV 感染者の結核死亡数（千人） | 206 | 5.9 | 4.6 | 3.7 | 24 | 5.1 | 248 |

Published in Global TB Control 2006 (www.who.int/tb/publications/global_report). Visit www.who.int/tb/country/tb_burden for 2000 estimates published in Corbett et al. 2003. Arch Intern Med 163: 1009–1021.

ました．2004 年には DOTS 戦略によって発見・治療された塗抹陽性患者は 209 万人であり，推定患者数の 53％にあたりました．しかし，DOTS で治った人は推定患者の 36％にとどまっていました．そこで結核高負担国ならびに DOTS 実施の遅れている国々に対して，「ストップ結核パートナーシップ」のスローガンのもとに，DOTS 戦略の拡大を図っています．感染症は国を超えて広がっていることから，2000 年の「九州・沖縄サミット」のときに「世界エイズ・結核・マラリア対策基金」が設立されました．また，2000 年 9 月ニューヨークで開催された国連ミレニアム・サミットで国連ミレニアム宣言が採択され，ミレニアム開発目標（MDGs）が定められました．この中で定められた 8 つの目標の中の 6 番目の目標の第 8 の課題項目として結核対策の推進が挙げられ，国際的に取り組まれることになりました．

## 欧米諸国の結核の動向

米国，英国は我が国よりはるかに結核の少ない国であります（表3）．しかし，1980 年代に米国の大都市は結核の再興に苦しみ，特にニューヨークにおける多剤耐性結核患者の増加はパニックにまでなりました．ニューヨークにおいては 1970 年代後半から結核対策に対する連邦政府，市の財政支援の大部分が削減されたために，結核対策のスタッフが減らされ，診療部門も縮小され，結核対策の基盤が弱体化していました．そこに HIV の流行，結核高罹患国からの移民の増加，経済の悪化が加わり結核再興につながっていきました．特に 1980 年代から 1990 年代初期にかけての多剤耐性結核の流行は一般市民を不安に落し入れました．そのために結核対策の予算が一気に増やされ，連邦政府，州，地方レベルの各段階の結核対策，院内感染対策，患者の Directly Observed Therapy（DOT）の強化がなされました[3]．1992 年から 2002 年の 10 年間には結核患者数が 45％ も減少し，結核罹患率は5にまで半減するというみごとな成果を上げました．英国も結核罹患率は我が国と比べて低い状況であったのでありますが，結核患者が 1988 年から増加に転じました．英国の結核患者は，ロンドン，中央部，北西部の都市部に 70％ 以上が集中しています．特にロンドンの罹患率は高く 41 で，全国の患者の 43％ がロンドンに集中して，他地域との格差は拡大傾向にあります．ロンドンの結核の特徴は，63％ が英国以外で生まれた人々であります．2002 年に前年と比べて英国の患者数がさらに 4％ 増加し，罹患率が 12.9 となりました．このため英国政府は，結核問題が放置のできない深刻な保健問題となってきたため，2004 年 10 月に新たな結核対策の行

表3 2004 年の主な先進国の結核推計値（WHO）

| 主な先進工業国 | 全結核推定数 | 罹患率 |
|---|---|---|
| スウェーデン | 390 | 4.3 |
| 米国 | 13,877 | 4.7 |
| カナダ | 1,663 | 5.2 |
| オーストラリア | 1,132 | 5.7 |
| イタリア | 4,093 | 7.1 |
| オランダ | 1,330 | 8.2 |
| ドイツ | 6,773 | 8.2 |
| 英国 | 7,101 | 11.9 |
| フランス | 7,411 | 12.3 |
| 日本 | 37,814 | 29.6 |
| 韓国 | 43,029 | 90.3 |
| 全世界 | 8,918,203 | 139.6 |

推計値は WHO により再計算されたものである．

動計画（Stopping Tuberculosis in England：An Action Plan）を策定しました[4]．また，感染症にかかわる保健医療システムも新たに再構築し，中央，地方レベルに新たな組織を設けて対策を強化し始めたことにより，ようやく結核罹患率の上昇傾向に歯止めがかかるようになってきました．ニューヨークやロンドンの事例は，世界の結核問題が解決しない中で結核対策の手をゆるめると結核の再興が起りうることを示すものであります[5]．

## 我が国の結核の現状

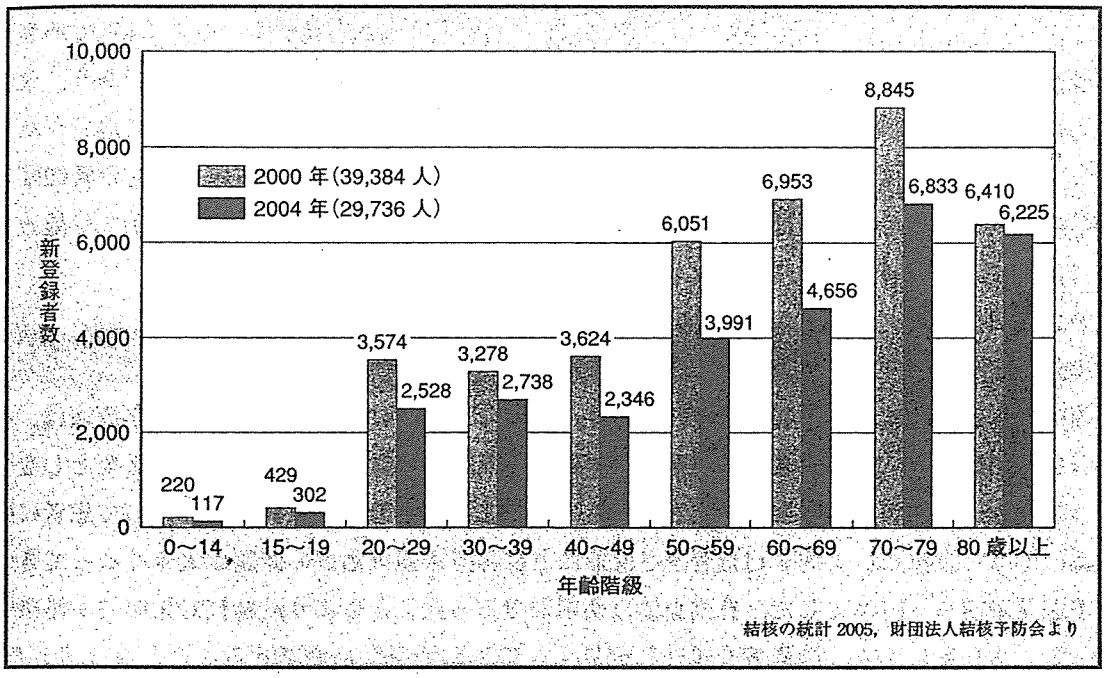
### 1．我が国の結核問題の特徴

我が国は欧米先進国と比べて結核の蔓延が遅れて生じました（表3）．そのために我が国の結核対策は高度経済成長，医療保障制度の確立の時期と重なり，また医療技術の点でも胸部X線写真による結核検診，抗結核薬，BCG 接種の登場と普及の時期と重なり，これらの対策技術を取り入れて社会を挙げて結核対策を進めることができました[6]．そのために我が国の結核罹患率は 1970 年代末までは急速に低下させることができました．その結果，若年者の大部分を未感染者にとどめることができていますが，結核が蔓延していた時代に青年期を迎えていた高齢者が平均寿命の延伸のため既感染者として多く存在し続けているという，結核事情が大きく異なる世代が同居する社会になりました．新登録患者をみますと 70 歳以上の者の割合は 40％，60 歳以上の者が 60％ を占めていることはこのような状況からであります（図1）．現在，結核塗抹陽性罹患率が減らない状況になっています．国民や医療関係者の中で結核の認識が低下してきたことがあると考えられます．このために 1999 年に現厚生労働省は結核緊急事態宣言を発令し注意を喚起しています．現在も1年間に新たに患者になる人は約3万人弱，死亡者は約2千人にものぼっています．また，HIV / AIDS は日本で増加の一途をたどっており，今のところ結核と AIDS の合併は大きな問題となっていませんが，将来は欧米社会におけるような課題となる可能性も考えられます．

### 2．都市問題としての結核

我が国の結核は，全国に均一に広く蔓延していた状況から，都市部を中心に高齢者などに患者が集中する状況に変化してきています．し

図1 我が国の 2000 年および 2004 年の年齢階級別結核新登録者数




かも，大都市部の特定地域と特定の社会階層の人々に集積する傾向となってきています[7]．この背景には都市部には日雇い労働者などの不安定就労者が多いことがあります．これらの人々は地域，職域の保健活動の狭間にあり，健康管理機会が乏しい状況にあり，しかも経済的に困窮しているために有症状時にも医療機関に早期に受診することができず，そのため発症から患者発見までの期間が長くなりがちであり，結核の大量排菌者，重症者，死亡者が多く，感染の悪循環を断ち切れていない状況にあります．しかも，我が国は経済発展して豊かな国となったことから，健康管理体制は勤務者や住民には細かな対策を実現することができていますが，結核患者が偏在化している流動性の高い，不安定就労者，不安定生活者に対する公衆衛生対策についてはむしろ弱体化している状況にあります．このために，このような人々が多く居住している都市部ほど結核問題の解決が難しい状況になっています．都道府県（指定都市を除く）と 12 指定都市の合計 59 自治体の結核新登録患者数を 2003 年の統計からみますと，大都市とその周辺の 11 地区で全国患者総数の半数が占められています．特に，東京都（特別区＋都下）が 12.7 %，大阪（府＋市）が 12.2 % であり，両都市

で我が国の結核患者の4分の1が占められています．罹患率では，大阪市 68.1，名古屋市 36.1，神戸市 36.1，東京都特別区 34.7 でありますが，東京都特別区の中では台東区 86，新宿区 60 などであり，大阪市においても西成区 293，浪速区 95 と罹患率が特に高い地域があります．また，東京都隣接の千葉県と埼玉県，名古屋市を含む愛知県などは罹患率は高くないですが，人口が多く患者の絶対数が多い地域であります[8]．

## 世界の結核対策と新たな戦略

1940 年代後半から 1950 年代前半にかけて登場した化学療法は，それまで結核治療の原則とされていた「入院」を必ずしも必要としないなど大きな変化をもたらしました．しかし，長期にわたって患者に服薬を自己管理させることに伴う不規則治療の問題が大きくなってきました．有効な治療薬剤がそろったことにより，結核対策の柱が感染性の患者を迅速に発見し，確実に治療し治癒させることとなりました．DOTS は現在世界で利用できる診断と治療の方法を利用し，どこの地域でも行える対策として確立されたものであります．HIV 関連結核や多剤耐性結核に対するために DOTS－Plus 戦略も進められています．しかし，世界で流行し続けている結核問題を劇的に解決していくためには DOTS 戦略だけでは力不足であり，新たな結核対策技術や方法の確立が待たれています[9]．発病を阻止する強力な発病予防ワクチンの開発，菌陰性患者に対しても使える簡便でより優れた診断検査方法の開発，有効な予防投薬の方法の確立や治療薬剤の開発などが必要であります[10][11]．また，結核対策を進めていくには，どのような保健医療体制で進めていくかも重要な課題であり，この点から，DOTS 戦略を進めるにあたり，その地域の保健医療サービスの提供者を総動員することが重要となってきています．つまり，多くの非政府系の保健サービス提供者，例えば私的医療施設，大学・研究施設，NGO のサービス，伝統的な医術者なども対策の協力者として位置づける必要性が重要とされています[12]．

## 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．

高鳥毛敏雄

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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.**

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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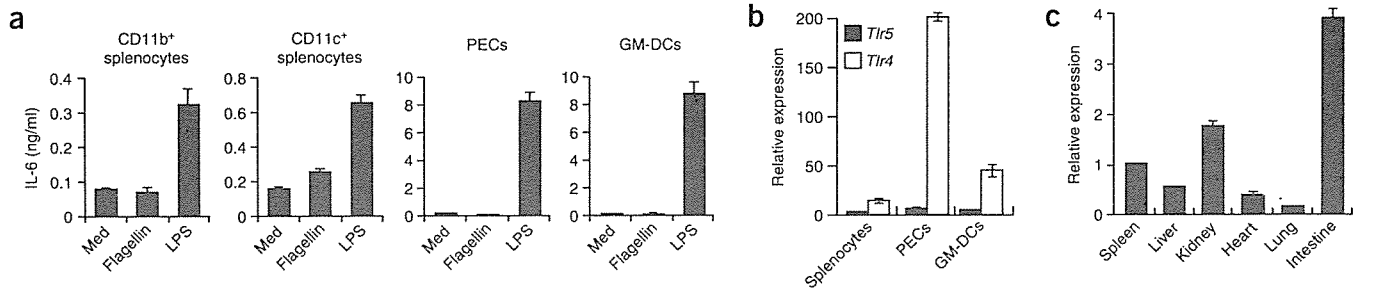


**Figure 1** Macrophages and conventional DCs are hyporesponsive to flagellin. (**a**) Enzyme-linked immunosorbent assay of IL-6 production by splenic CD11b$^+$ and CD11c$^+$ cells, GM-DCs and peritoneal macrophages (PECs) from C57BL/6 mice. Cells were cultured with medium only (Med), flagellin (1 μg/ml)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

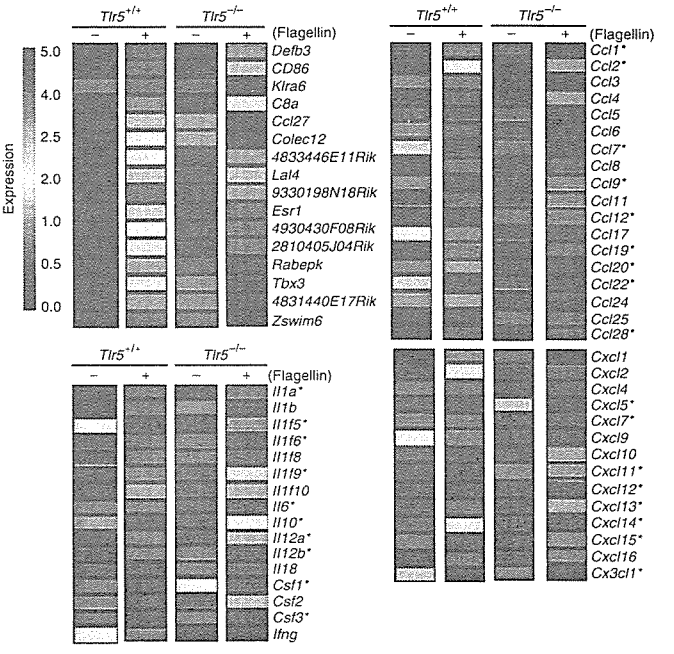


**Figure 2** Gene expression induced by flagellin stimulation in IECs. Microarray analysis of IECs from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice stimulated with medium alone (–) or 1 μg/ml of flagellin (+). *, genes judged as being statistically undetectable at all time points. There is flagellin-induced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

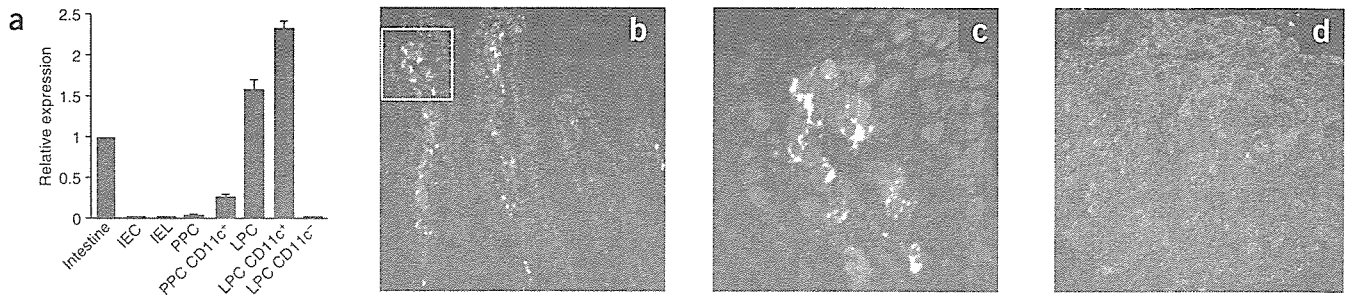


Figure 3 TLR5 is highly expressed on CD11c$^+$ LPCs. (a) Quantitative real-time PCR of *Tlr5* expression by the intestine (far left) and by various cell types of C57BL/6 mice. IEL, intestinal epithelial lymphocyte. Data are mean ± s.d. of triplicate samples from one representative of three independent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

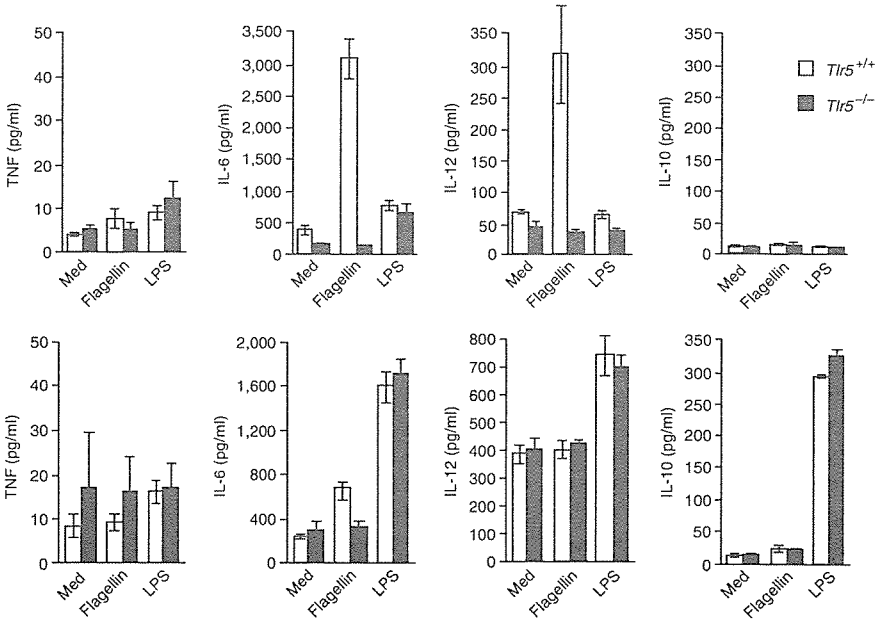


Figure 4 TLR5-mediated CD11c$^+$ LPC cytokine production. Enzyme-linked immunosorbent assay of cytokine production by CD11c$^+$ LPCs (top) and PPCs (bottom) from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice. Cells were cultured with medium only, flagellin (1 μg/ml) or LPS (100 ng/ml). Data are mean ± s.d.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

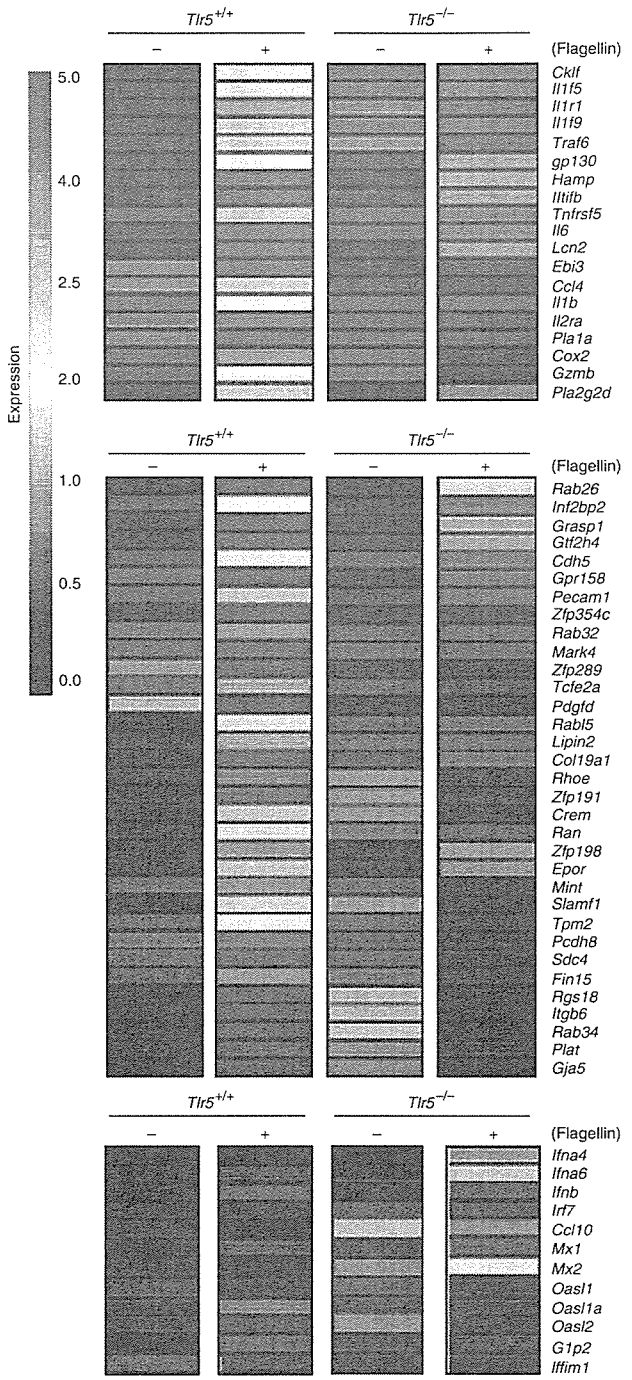


**Figure 5** Flagellin-induced gene expression in CD11c$^+$ LPCs. Microarray analysis of CD11c$^+$ LPCs from *Tlr5*$^{+/+}$ and *Tlr5*$^{-/-}$ mice left unstimulated (–) or stimulated with 1 μg/ml of flagellin (+). Upregulated genes encode cytokines (*Il6, Il1f5, Il1f9, Il1b, Ebi3* and *Iltifb*), cytokine 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 ($5 \times 10^8$ 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

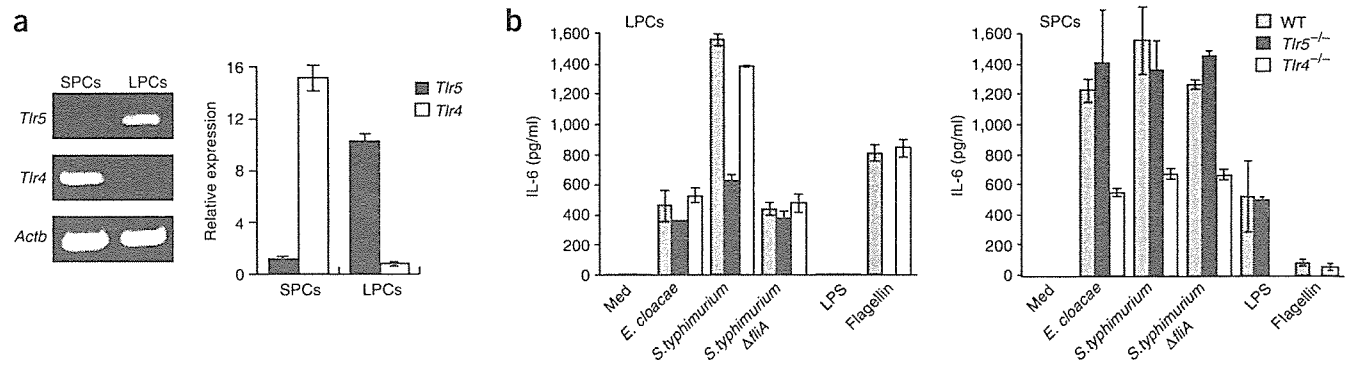


**Figure 6** CD11c$^+$ LPCs detect pathogenic bacteria via TLR5. (**a**) Quantitative real-time PCR of *Tlr5* and *Tlr4* expression in CD11c$^+$ SPCs and CD11c$^+$ LPCs of C57BL/6 mice. *Actb* encodes β-actin (loading control). Graphed data are mean ± s.d. of triplicate samples from one representative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

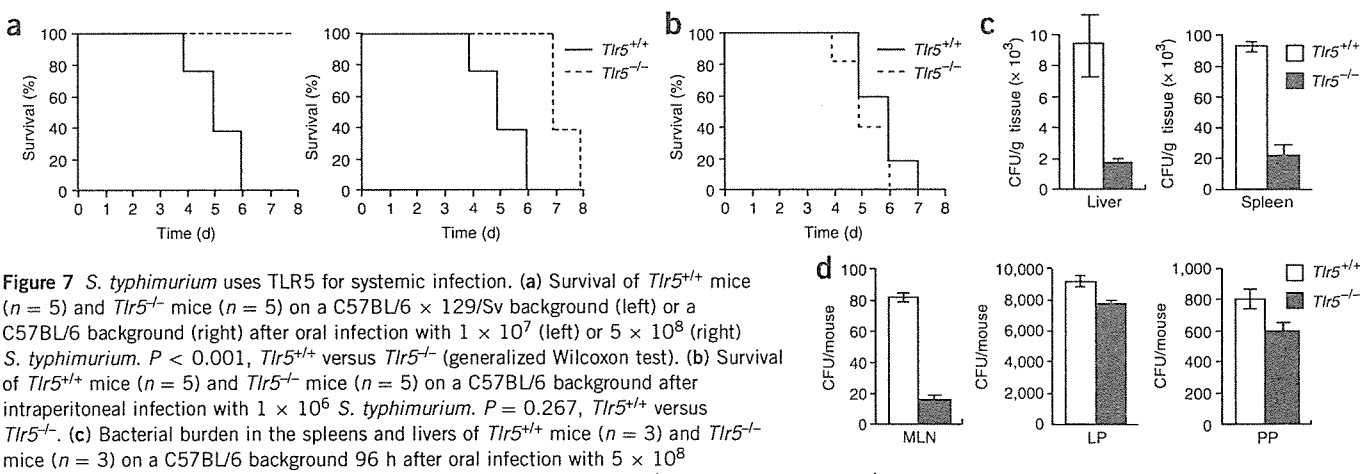


**Figure 7** *S. typhimurium* uses TLR5 for systemic infection. (**a**) Survival of *Tlr5*$^{+/+}$ mice ($n = 5$) and *Tlr5*$^{-/-}$ mice ($n = 5$) on a C57BL/6 × 129/Sv background (left) or a C57BL/6 background (right) after oral infection with $1 \times 10^7$ (left) or $5 \times 10^8$ (right) *S. typhimurium*. $P < 0.001$, *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 $1 \times 10^6$ *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 $5 \times 10^8$ *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 $5 \times 10^8$ *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 ($5 \times 10^4$ 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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# 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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[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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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-γR$^{-/-}$ mice by passages every other week (21). For in vitro experiments, macrophages or DCs ($5 \times 10^4$) were infected with $5 \times 10^4$ 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 $2 \times 10^3$ or $1 \times 10^4$ 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 ($5 \times 10^4$) were infected with $5 \times 10^4$ *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,

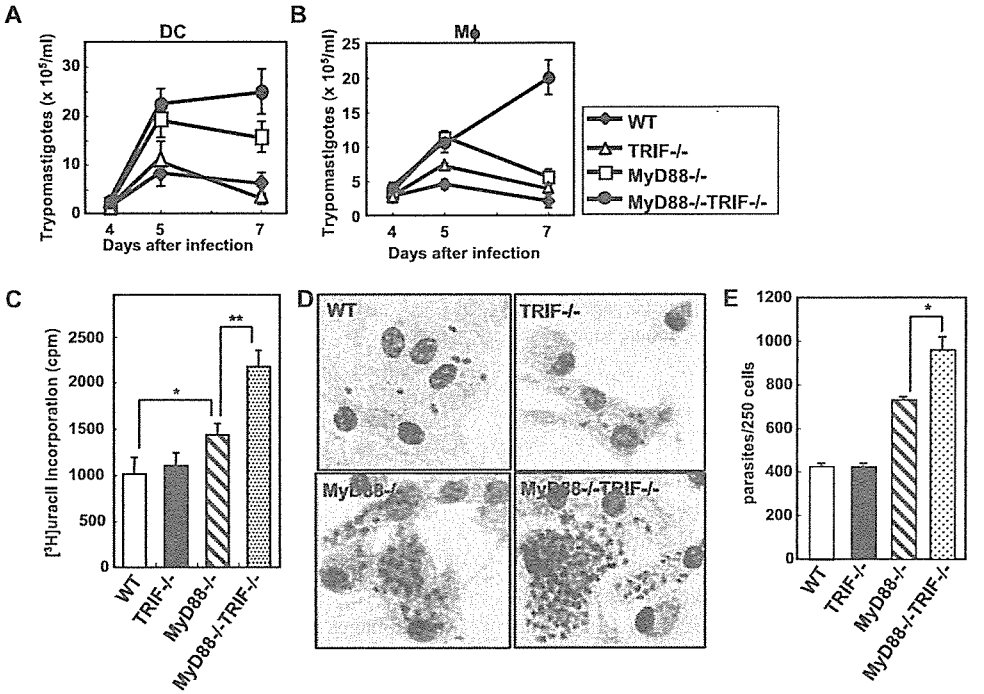


**FIGURE 1.** Defective *T. cruzi* clearance in MyD88$^{-/-}$TRIF$^{-/-}$ DCs and macrophages. Bone marrow-derived DCs (*A*) or peritoneal macrophages (M$\phi$) (*B*) from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$TRIF$^{-/-}$ mice were seeded onto 96-well plates, and infected with *T. cruzi* 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-$\alpha$ 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-$\beta$ (9, 10). Therefore, we analyzed *T. cruzi*-induced gene expression focusing on IFN-$\beta$ 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-$\alpha$ and IL-12p40 mRNAs (Fig. 3*A*). In contrast, both MyD88$^{-/-}$ and MyD88$^{-/-}$TRIF$^{-/-}$ macrophages showed defective induction of TNF-$\alpha$ and IL-12p40. Expression of the mRNAs for IFN-$\beta$ 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-$\alpha$4 mRNA was not observed in any of the macrophage and DC genotypes (data not shown). In MyD88$^{-/-}$ 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-$\beta$ and IFN-inducible genes during the infection. Furthermore, MyD88$^{-/-}$TRIF$^{-/-}$ cells displayed defective expression of all these genes.

### *IFN-$\beta$-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-$\beta$ and IFN-inducible genes. Therefore, we next addressed whether IFN-$\beta$ 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-$\beta$ 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-$\beta$ Ab. In contrast, anti-IFN-$\beta$ Ab addition dramatically increased the intracellular growth of *T. cruzi* in MyD88$^{-/-}$ macrophages. These findings indicate the possible involvement of IFN-$\beta$ 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-$\beta$ 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

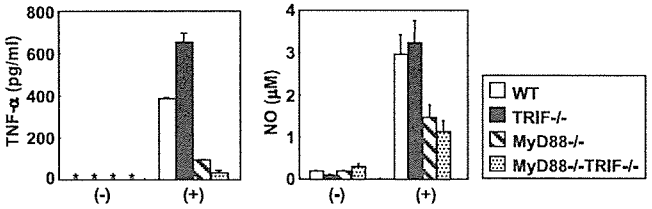


**FIGURE 2.** Defective production of TNF-α and NO in *T. cruzi*-infected MyD88$^{-/-}$ macrophages. Peritoneal macrophages from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$TRIF$^{-/-}$ mice were infected with (+) or without (−) *T. cruzi* for 6 h, washed to remove the extracellular parasites, and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

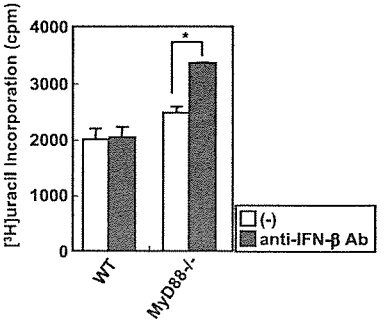


**FIGURE 4.** Effect of an anti-IFN-β neutralizing Ab on *T. cruzi* growth in macrophages. Peritoneal macrophages from WT or MyD88$^{-/-}$ mice were infected with *T. cruzi* for 6 h in the presence or absence of an anti-IFN-β neutralizing Ab, washed, and cultured in the presence of [$^3$H]uracil for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

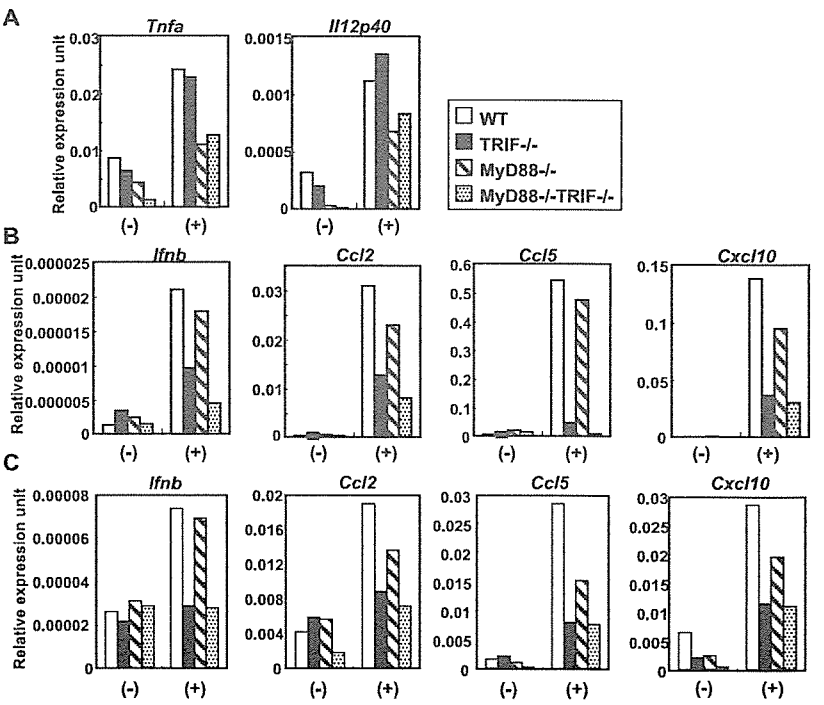


**FIGURE 3.** *T. cruzi*-induced expression of inflammatory genes in macrophages and DCs. *A*, Peritoneal macrophages from WT, TRIF$^{-/-}$, MyD88$^{-/-}$, or MyD88$^{-/-}$TRIF$^{-/-}$ mice were cultured in the presence (+) or absence (−) of *T. cruzi* for 6 h. Total RNA was then extracted and analyzed 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.

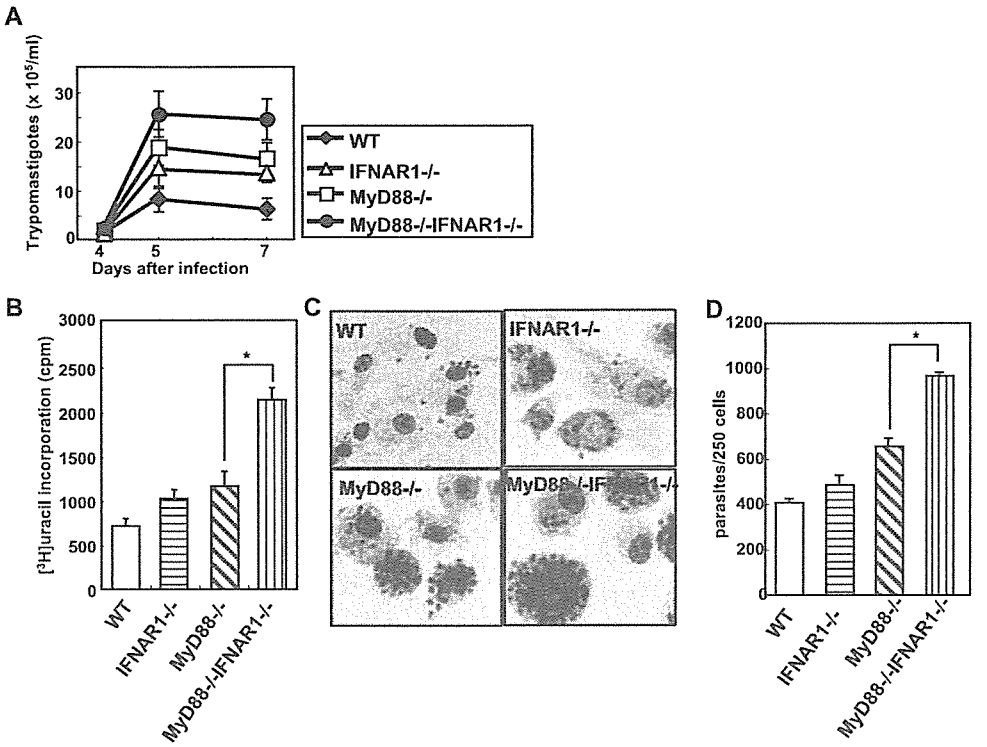


**FIGURE 5.** Increased *T. cruzi* growth in MyD88$^{-/-}$IFNAR1$^{-/-}$ macrophages. *A*, Bone marrow-derived macrophages from WT, MyD88$^{-/-}$, IFNAR1$^{-/-}$, or MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were infected with *T. cruzi* for 6 h, washed to remove the extracellular parasites, and cultured for 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-$\beta$ 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-$\beta$-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

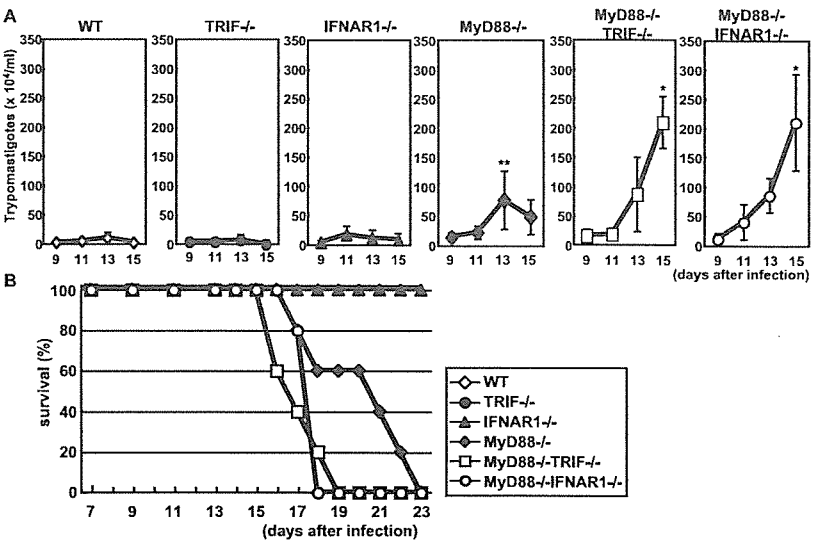


**FIGURE 6.** High-sensitivity MyD88$^{-/-}$IFNAR1$^{-/-}$ mice to *T. cruzi* infection. WT ($n = 9$), TRIF$^{-/-}$ ($n = 10$), IFNAR1$^{-/-}$ ($n = 10$), MyD88$^{-/-}$ ($n = 5$), MyD88$^{-/-}$TRIF$^{-/-}$ ($n = 5$), and MyD88$^{-/-}$IFNAR1$^{-/-}$ ($n = 5$) mice were i.p. infected with $1 \times 10^4$ *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.

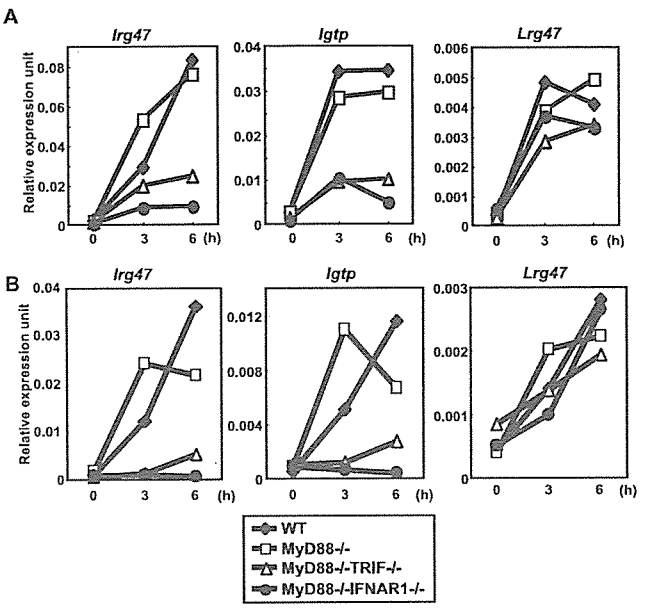


**FIGURE 7.** Impaired expression of IRG47 in *T. cruzi*-infected MyD88$^{-/-}$TRIF$^{-/-}$ mice. Bone marrow-derived macrophages (*A*) or DCs (*B*) from WT, MyD88$^{-/-}$, MyD88$^{-/-}$TRIF$^{-/-}$, or MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were infected with *T. cruzi* for 3 or 6 h. Next, total RNA was extracted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

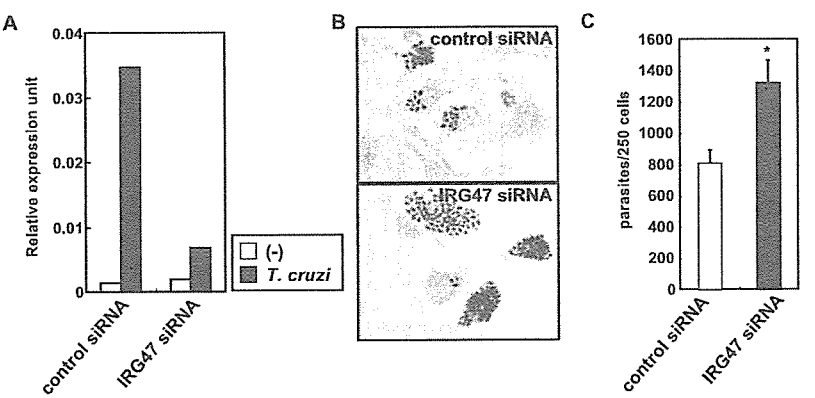


**FIGURE 8.** IRG47 mediates antitrypanosomal activity in MyD88$^{-/-}$ mice. *A*, Bone marrow-derived macrophages were transfected with IRG47 or control siRNA and cultured for 18 h. The cells were then infected with *T. cruzi* for 6 h and analyzed for the expression of IRG47 mRNA by quantitative real-time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