TLR5-Deficient Mice Lack Basal Inflammatory and Metabolic Defects but Exhibit Impaired CD4 T Cell Responses to a Flagellated Pathogen

Shirdi E. Letran, Seung-Joo Lee, Shaikh M. Atif, Adriana Flores-Langarica, Satoshi Uematsu, Shizuo Akira, Adam F. Cunningham and Stephen J. McSorley

_J Immunol_ 2011; 186:5406-5412; Prepublished online 30 March 2011; doi: 10.4049/jimmunol.1003576

http://www.jimmunol.org/content/186/9/5406

References  This article cites 38 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/186/9/5406.full#ref-list-1

Why _The JI_? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

Subscription  Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
TLR5-Deficient Mice Lack Basal Inflammatory and Metabolic Defects but Exhibit Impaired CD4 T Cell Responses to a Flagellated Pathogen


TLR5-deficient mice have been reported to develop spontaneous intestinal inflammation and metabolic abnormalities. However, we report that TLR5-deficient mice from two different animal colonies display no evidence of basal inflammatory disease, metabolic abnormalities, or enhanced resistance to *Salmonella* infection. In contrast, the absence of TLR5 hindered the initial activation and clonal expansion of intestinal flagellin-specific CD4 T cells following oral *Salmonella* infection. Together, these data demonstrate that a basal inflammatory phenotype is not a consistent feature of TLR5-deficient mice and document a novel role for TLR5 in the rapid targeting of flagellin by intestinal pathogen-specific CD4 T cells. *The Journal of Immunology*, 2011, 186: 5406–5412.

Toll-like receptors allow host recognition of microbe-associated molecular patterns and rapid initiation of an inflammatory response to invading pathogens (1, 2). Recent data also indicate that these innate immune receptors detect microbe-associated molecular patterns expressed by commensal organisms, and this recognition can be vitally important for maintaining immune homeostasis, particularly in the intestine (3). Lack of an individual TLR or adaptor molecules required for TLR signaling can therefore disrupt immune homeostasis and increase susceptibility to inflammatory diseases (4, 5).

TLR5 specifically recognizes flagellin, the major protein constituent of bacterial flagella, a conserved microbial structure known to be required for bacterial directed motility (6–10). Ligation of TLR5 initiates an innate immune response that is characterized by host cell production of inflammatory chemokines and cytokines, requiring MyD88, MAPK, and NF-kB activation (9, 11, 12). Bacterial flagellins can also be recognized by non-TLR host cytosolic receptors, leading to caspase-1 activation and IL-1β secretion (13), highlighting the importance of flagellin recognition for antibacterial immunity. The initial reports describing TLR5-deficient mice confirmed that TLR5 is required for the rapid inflammatory response induced after injection of soluble flagellin (14, 15) and also suggested that TLR5-deficient mice are more resistant to some flagellated pathogens (14), but also more susceptible to others (16).

In addition to this well-studied interaction of flagellin with host innate immune responses, flagellin also happens to be a protein Ag that is specifically targeted by the adaptive immune system during bacterial infection and inflammatory disease (17–19). Thus, the potential exists that host expression of a flagellin receptor could serve to modulate Ag-specific CD4 T cell responses to flagellin peptides (7). However, one recent study has argued against this possibility because flagellin-specific IgG responses were found to develop normally in the absence of TLR5 (20).

Other reports have suggested that TLR5 might play an important role in immune homeostasis, particularly in regulating host responses to intestinal microbial flora (9, 21). For example, TLR5-deficient mice were reported to develop spontaneous colitis due to an inflammatory response that was initiated by enteric flora and required TLR4 expression (22). Furthermore, TLR5-deficient mice were also reported to be innately resistant to infection with *Salmonella typhimurium* (23). Indeed, this resistance was most likely due to the basal intestinal inflammatory response described in TLR5-deficient mice, because it did not require host recognition of *Salmonella* flagellin (23). More recently, a rederived line of TLR5-deficient mice was reported to suffer from a profound metabolic syndrome, including hyperphagia, hypertension, resistance to insulin, and increased fat-pad mass (24). Together, these various reports strongly suggest that TLR5-mediated homeostatic responses to natural enteric flora are vitally important for maintaining overall host metabolism and also avoiding the initiation of harmful intestinal inflammation.

In this study, we have used a range of assays to examine several important metabolic and intestinal inflammatory variables and report the absence of basal inflammatory or metabolic defects in TLR5-deficient mice from two independent animal facilities. In marked contrast, TLR5-deficient mice displayed a prominent defect in the activation of flagellin-specific mucosal CD4 T cell responses following oral infection with flagellated *Salmonella*. Our data therefore demonstrate that TLR5 plays a crucial role in shaping the adaptive immune response to a flagellated pathogen.
but suggest that any role for TLR5 in initiation of basal inflammatory or metabolic defects is likely to require unique floral components or other additional factors.

Materials and Methods

Mouse and bacterial strains

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME) and used at 6–20 wk of age. TLR5-deficient mice were bred at the University of Minnesota. C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME) and used at 6–20 wk of age. TLR5-deficient mice were bred at the University of Minnesota and were bred at the University of Minnesota. All mice were cared for in accordance with University of Minnesota Research Animal Resource guidelines and UK Home Office approval. Salmonella strain SL1344 was kindly provided by Dr. D. Xu (University of Glasgow, Glasgow, U.K.). LPS-deficient S. typhimurium X4700 was generously provided by Dr. R. Curtiss (Arizona State University).

Examination of basal inflammatory and metabolic defects

Cohorts of wild-type and TLR5-deficient mice were weighed weekly to examine overall body mass. At ~10 or 20 wk of age, blood was collected from groups of sex-matched wild-type and TLR5-deficient mice before cohorts from each group were sacrificed and organs harvested. Cecum and colon were observed closely for intestinal bleeding and overt signs of shrinkage or swelling before being weighed along with harvested spleens, livers, and fat pads. After weighing, spleens and livers were homogenized and whole organs plated onto Luria-Bertani agar to detect bacterial growth. Serum amyloid A (SAA) levels were examined in the sera using an ELISA kit according to the manufacturer’s instructions (Biosource International). Insulin and glucose levels were measured using commercially available assays and following the manufacturer’s instructions.

Purification and injection of flagellin

Flagellin was purified from S. typhimurium (X4700) using a modified acid-shock protocol (29, 30). Bacteria were grown at 37°C without shaking before being washed and resuspended in PBS/HCl (pH 2) for 30 min at room temperature. Bacterial supernatants were collected, and ultracentrifugation and ammonium sulfate precipitation were used to purify flagellin. Residual LPS was removed by serial passage through multiple detoxigel columns (Fierce Biotechnology). Silver-stained SDS-PAGE gels were used to confirm purity of flagellin preparations, and each batch of flagellin was found to be LPS-free using the Limulus assay. Groups of mice were injected i.v. with 10 µg Salmonella flagellin and serum collected to examine inflammatory cytokine production.

Salmonella infection and bacterial counts

S. typhimurium SL1344 or BRD509 (AroA− ΔSal1) were grown overnight in Luria-Bertani broth without shaking and diluted in PBS after estimation of bacterial concentration using a spectrophotometer. Mice were infected orally with ∼5 × 10⁹ SL1344 after administration of 0.1 ml 5% sodium bicarbonate. In all experiments, the actual bacterial dose administered was confirmed by plating serial dilutions of the original culture onto MacConkey agar plates. Mice were monitored daily for signs of infection and determined to be moribund if unresponsive to gentle prodding, at which point they were euthanized as stipulated by our animal care protocol. To determine bacterial colonization in vivo, Peyer’s patches, mesenteric lymph nodes (MLN), and spleens from infected mice were homogenized in PBS, and serial dilutions were plated onto MacConkey agar plates. After overnight incubation at 37°C, bacterial plates were counted and bacterial burdens calculated for each individual organ.

TCR transgenic adoptive transfers and analysis

Spleen and lymph node cells (inguinal, axillary, brachial, cervical, mesenteric, and peri-aortic) were harvested from SM1 mice, and a single-cell suspension was generated. An aliquot of this sample was stained using Abs to CD4 and TCR Vβ2 to determine the percentage of TCR transgenic cells. Volumes were adjusted accordingly, and 1 × 10⁷–1 × 10⁸ SM1 were injected i.v. into recipient C57BL/6 mice. At various time points post-infection, Peyer’s patches, MLN, and spleens were harvested and a single-cell suspension generated in complete EHAA medium. Samples were

![FIGURE 1](http://www.jimmunol.org/)

Lack of basal inflammatory phenotype in TLR5-deficient mice. Age-matched wild-type and TLR5-deficient mice were euthanized at 10–12 wk and examined for evidence of basal inflammation. A. Photograph showing intestinal length from wild-type and TLR5-deficient mice. B–D. Spleen, colon, and cecum weight of wild-type and TLR5-deficient mice. Graphs show mean organ weight ± SEM. E. SAA levels for male wild-type and TLR5-deficient mice were determined by ELISA. Graph shows mean SAA levels ± SEM for 9–11 mice per group. No significant difference in SAA levels was found when comparing wild-type and TLR5-deficient mice by unpaired t test (p = 0.1627).
incubated on ice for 30 min in F, block containing FITC-, PE-, PE-Cy5-, or allophycocyanin-conjugated Abs specific for CD4, CD11a, CD25, CD69, CD45.1, or CD90.1 (eBioscience and BD Biosciences). After staining, cells were fixed using paraformaldehyde and examined by flow cytometry using an FACSCanto (BD Biosciences). All flow data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

**Purification of dendritic cells and in vitro stimulation of T cells**

Spleens were harvested from mice and incubated with collagenase D (37°C for 20 min) and EDTA to liberate dendritic cells. Magnetic anti-CD11c microbeads and multiple passes through selection columns (Miltenyi Biotec, Auburn, CA) were used to isolate CD11c+ spleen dendritic cells to 85–95% purity. Purified dendritic cells (1 × 10^6/well) were washed and placed in culture with SM1 T cells (1 × 10^5/well) plus titrated numbers of attenuated bacteria (BRD509) for 30 min. Cells were then harvested and washed before being cultured for 6 h in medium containing antibiotics. Individual wells were harvested and stained for Abs specific to CD4 and CD90.1 (to detect SM1 T cells) and surface activation molecules CD25 and CD69. Samples were acquired using an FACSCanto flow cytometer (BD Biosciences) and data analyzed using FlowJo (Tree Star).

**Statistical analysis**

Data were first determined to be normally distributed and differences between groups examined using InStat (GraphPad, La Jolla, CA). Data in each group were compared using an unpaired t test and considered significantly different with a p value <0.05.

**Results**

**TLR5-deficient mice do not develop basal inflammatory or metabolic defects**

A recent study reported that 10–12% of TLR5-deficient mice developed spontaneous rectal prolapse and also had lower body weight, indicating the development of colitis (22). In the same study, analysis of the TLR5-deficient mice lacking prolapse found that 20% of these mice developed intestinal bleeding, ~30% had increased colon and spleen weight, and 40% had high levels of SAA (22). In an effort to determine whether these findings are consistent among TLR5-deficient mouse colonies, we examined these variables in a TLR5-deficient colony at the University of Minnesota. Surprisingly, our analysis demonstrated that TLR5-deficient mice displayed no evidence of rectal prolapse, intestinal bleeding, contracted cecum, increased colon or spleen weight, or reduced cecum weight when compared with wild-type mice (Fig. 1 and data not shown). In addition, we found no evidence of rectal prolapse or increased spleen weight in an independent TLR5-deficient colony housed at the University of Birmingham (data not shown). SAA levels were also found to be within a similar range for TLR5-deficient and wild-type mice at 10 and 20 wk of age (Fig. 1E and data not shown). Furthermore, bacteria were absent from the spleen and liver of TLR5-deficient mice at Minnesota and Birmingham (data not shown), despite the fact that low-level bacterial colonization in these organs was previously reported (22).

Given the previous correlation of basal intestinal inflammation and nonspecific resistance to *Salmonella* (22, 23), we also examined the susceptibility of these mice to *Salmonella* infection and monitored bacterial growth in the spleen. Consistent with our inability to detect a basal inflammatory state in TLR5-deficient mice, enhanced resistance to primary *Salmonella* infection was not observed, as measured by prolonged survival or reduced colonization postinfection (Fig. 2). Together, these data demonstrate that a predisposition to spontaneous colitis and nonspecific resistance to *Salmonella* infection is not a constant feature of TLR5-deficient mouse colonies.

A more recent study reported that mice lacking TLR5 expression display a wide range of clinical and metabolic abnormalities that bear some similarity to human metabolic syndrome (24). In this particular study, TLR5-deficient mice were found to have a 20% increase in body mass, significantly larger fat pads, elevation of blood glucose levels, and a substantial increase in basal insulin levels (24). Again, we examined many of these features in our two different TLR5-deficient colonies but were unable to detect significant differences in body mass, blood glucose levels, or basal insulin levels compared with wild-type mice (Fig. 3 and data not shown). At 20 wk of age, a small increase in fat pad size was observed in male TLR5-deficient mice (Fig. 3D). However, this was not detected in female mice and did not appear to correlate with changes in any other measured parameter. Therefore, the development of basal inflammatory or metabolic disregulation is not a consistent feature of TLR5-deficient mouse colonies.
TLR5 is necessary for early targeting of intestinal CD4 T cell responses to a flagellated pathogen

Bacterial flagellin is a major antigenic target during infection and inflammatory disease (18, 19, 31–33), and it was therefore of interest to examine whether TLR5 deficiency had any effect on flagellin-specific T cell activation postinfection with a flagellated pathogen. We previously generated a TCR transgenic mouse line using the TCR \( \alpha \)-and \( \beta \)-chains of a T cell clone specific for *Salmonella* flagellin 427–441 in the context of I-\( \beta \) (18, 25). Naive peripheral SM1 CD4 T cells can be used to monitor host T cell responses to bacterial flagellins in vitro and in vivo (7, 34, 35).

Wild-type and TLR5-deficient mice were adoptively transferred with flagellin-specific SM1 T cells, infected orally with *Salmonella*, and the activation and the expansion of SM1 T cells was directly examined in mucosal lymphoid tissues. As reported previously (36), almost all SM1 T cells in the Peyer’s patch of wild-type mice were activated to express increased levels of CD69 within 6 h of oral infection, and similar activation was detected a few hours later in the MLN (Fig. 4). In marked contrast, only 25–30% of SM1 T cells were activated in the Peyer’s patch of TLR5-deficient mice 6 h postinfection (Fig. 4). Furthermore, lower peak activation was detected at all time points examined in the Peyer’s patches and MLN of infected TLR5-deficient mice (Fig. 4). Similar deficient responses were also noted when CD25 was used as a marker of T cell activation (data not shown). Together, these data demonstrate that TLR5 plays an obligatory role in the targeting of flagellin by intestinal *Salmonella*-specific CD4 T cells during oral infection. As SM1 T cells were ultimately activated in the Peyer’s patch and MLN of infected TLR5-deficient mice, we also examined whether delayed early activation affected clonal expansion of these cells at later time points. Indeed, clonal expansion of SM1 T cells was significantly lower in the Peyer’s patch and MLN of TLR5-deficient mice compared with wild-type mice 3 d after oral infection (Fig. 5). Thus, TLR5 expression is required for the development of robust intestinal CD4 T cell responses to flagellin.

We recently reported that TLR5 is able to function as an endocytic receptor enhancing Ag presentation of flagellin epitopes in an MyD88-independent manner after flagellin immunization (34). To determine if MyD88 was required for early SM1 T cell activation during *Salmonella* infection, we compared SM1 T cell activation in infected wild-type, MyD88-, and TLR5-deficient mice. Reduced SM1 T cell CD69 activation was again observed at all time points in the Peyer’s patch of TLR5-deficient mice (Fig. 4D). In contrast, similar activation was detected in wild-type and MyD88-deficient mice, although CD69 activation was lower in MyD88-deficient mice at the 6 h time point (Fig. 4D). In marked contrast, SM1 CD69 expression was reduced in the MLN of both infected TLR5- and MyD88-deficient mice (Fig. 4E). These data suggest that TLR5 can enhance *Salmonella* flagellin-specific T cell responses in the Peyer’s patch but may do so in an MyD88-independent manner, whereas MyD88 signaling is likely to be important in the MLN.

**TLR5-deficient dendritic cells do not activate flagellin-specific CD4 T cells postinfection**

Given the differing results in the Peyer’s patch and MLN postinfection, we decided to directly examine whether purified TLR5-
and MyD88-deficient dendritic cells were capable of activating SM1 T cells in vitro in response to live Salmonella. As expected, dendritic cells from wild-type mice were able to activate SM1 T cells in vitro to increase expression of CD69 and CD25 (Fig. 6 and data not shown). However, TLR5-deficient dendritic cells poorly activated SM1 T cells in response to Salmonella infection and only at high multiplicities of infection (MOIs) (Fig. 6A). In contrast, dendritic cells from MyD88-deficient mice activated SM1 T cells to a similar degree and at similar MOIs as with wild-type dendritic cells (Fig. 6C). Thus, TLR5 expression is required to fully activate SM1 T cells in response to live bacteria in vitro, whereas MyD88 expression is dispensable.

### Discussion

In contrast to previous observations (22–24), we were unable to detect any evidence of basal intestinal inflammation or metabolic irregularities in TLR5-deficient mice in two different animal facilities. The TLR5-deficient colony in Minnesota has been housed for >2 y and never displayed any evidence of rectal prolapse or...
Increased weight gain. Interestingly, both the TLR5-deficient colonies in Minnesota and Birmingham were derived from the same TLR5-deficient mouse line that was used in previous reports describing basal inflammation, and the genetic disruption is therefore identical. The most likely explanation for the discrepancy in TLR5-deficient basal disease is a difference in the enteric flora between institutional animal facilities or differences acquired during rederivation of these animals. Indeed, the basal metabolic deficits reported for TLR5-deficient mice were found to be alleviated by antibiotic treatment (24), indicating that bacterial flora is likely to drive this particular pathology. Thus, our data are still consistent with the possibility that TLR5-deficient mice have increased susceptibility to developing basal intestinal inflammatory defects, depending on the composition of the local enteric flora. Most importantly, however, our data demonstrate that these very obvious and severe inflammatory and metabolic deficiencies are not an obligatory phenotype of this particular mouse strain and require factors that are not present in animal facilities in Minnesota or Birmingham. Additional research will be required to identify the causative agent of this interesting and important process and determine the actual penetrance of a basal inflammatory phenotype in TLR5-deficient mice housed at other institutions.

Our primary infection experiments using TLR5-deficient mice also failed to demonstrate any substantial difference in the resistance of these mice to Salmonella. This finding is in broad agreement with previous studies in which no difference in susceptibility to Salmonella infection was observed following i.p. infection (14, 15), but differs markedly from studies using the oral infection route (23). However, it is very likely that the absence of a basal inflammatory defect in the intestine of our mouse colony allows a more accurate assessment of the role of TLR5 in resistance to Salmonella infection, and we conclude therefore that TLR5 does not play a major role in initial innate immune defense against this particular pathogen. The presence of a basal inflammatory response might also explain the discrepancy between our data and a recent report examining flagellin-specific adaptive immune responses in TLR5-deficient mice (20). This study concluded that TLR5-deficient mice display no significant deficiency in the induction of flagellin-specific adaptive responses, whereas our data suggest a significant deficiency exists after oral infection with a flagellated pathogen.

Our examination of adaptive immune responses in Salmonella-infected mice demonstrate that initial activation of flagellin-specific CD4 T cell responses is significantly delayed in the intestine of TLR5-deficient mice. We previously reported that very early activation of Peyer’s patch CD4 T cells requires the mobilization of CCR6+ dendritic cells to the epithelial layer, presumably to capture invading bacteria and process Ag (36). Given our current data, this early mobilization of APC may actually be driven by inflammatory signals that require TLR5 ligation at the epithelial surface. Indeed, flagellin stimulation is known to cause the production of CCL20 (37), the chemokine ligand for CCR6 (38). However, given the fact that T cell activation was lower in the Peyer’s patch of TLR5-deficient versus Myd88-deficient mice and during in vitro stimulation assays, we prefer an alternative explanation, namely that TLR5 simply functions as an endocytic receptor to enhance flagellin processing by mucosal APC in vivo. Indeed, we recently reported that TLR5 can function as an endocytic receptor after immunization with purified bacterial flagellin. If this pathway is also important in driving flagellin-specific T cell responses to oral infection, then TLR5 expression by intestinal APC should be required for directing the early adaptive response to flagellin. However, it is currently unclear whether Peyer’s patch or MLN dendritic cell subsets actually express TLR5. A recent report demonstrated that TLR5 expression by APC is required for the adjuvant effect of flagellin after immunization (39), thus supporting some aspects of our proposed model. However, irrespective of whether this model of TLR5 scavenging by APC during infection is correct, our data conclusively demonstrate that TLR5 expression is required for robust flagellin-specific CD4 T cell activation in the gut mucosa during early oral infection.

In conclusion, we report that TLR5-deficient mice do not develop basal inflammatory or metabolic defects or enhanced resistance to Salmonella infection. In contrast, TLR5 plays an obligatory role in directing CD4 T cell targeting to bacterial flagellins during oral infection with a flagellated pathogen.

Acknowledgments

We thank the laboratory of Dr. S. Way for helpful discussions in completion of these experiments.

Disclosures

The authors have no financial conflicts of interest.

References