

# Toll-Like Receptor 5-Deficient Mice Have Dysregulated Intestinal Gene Expression and Nonspecific Resistance to *Salmonella*-Induced Typhoid-Like Disease<sup>∇†</sup>

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Received 7 November 2007/Returned for modification 14 December 2007/Accepted 28 December 2007

The recognition of flagellin by Toll-like receptor 5 (TLR5) is the dominant means by which model intestinal epithelia activate proinflammatory gene expression in response to *Salmonella enterica*. The role of the flagellin-TLR5 interaction in vivo has been addressed primarily via studies that use flagellar mutants. Such studies suggest that host recognition of flagellin promotes rapid neutrophil recruitment that protects the host from this pathogen. However, these works do not directly address the role of TLR5 and are subject to the caveat that flagellar mutations may broadly affect *Salmonella* gene expression. Thus, we examined the role of the flagellin-TLR5 interaction via the use of TLR5-deficient (TLR5KO) mice. We utilized both the traditional model of murine *Salmonella* infection, wherein low-dose oral infection of mice with *Salmonella enterica* subsp. *enterica* serovar Typhimurium results in systemic typhoid-like disease, and a more recently characterized model in which mice are pretreated with streptomycin to result in gut-restricted acute enteritis. In the enteritis model, TLR5KO mice had more severe gut pathology, thus “phenocopying” previous results obtained with *Salmonella* mutants. In contrast, TLR5KO mice were resistant to *Salmonella*-induced typhoid-like disease. However, such resistance was not specific for flagellated serovar Typhimurium, but rather, TLR5KO mice were also resistant to challenges by flagellin-deficient serovar Typhimurium. Such resistance associated with elevations in the microbiota was ablated by antibiotic pretreatment and correlated with basal elevations in intestinal host defense gene expression. All together, these results indicate that the resistance of TLR5KO mice to *Salmonella*-induced typhoid-like illness resulted from alterations in their basal phenotype rather than from the lack of TLR5 ligation during the infection per se.

The recognition of conserved microbial products by Toll-like receptors (TLR) is generally thought to protect the host against potentially pathogenic infections. However, in some instances, the loss of a specific TLR results in protection against a particular pathogen. For example, mice lacking the double-stranded RNA receptor TLR3 fare better when challenged with West Nile and influenza viruses (13). Analogously, it has recently been reported that mice lacking the flagellin receptor TLR5 are relatively resistant to the systemic typhoid-like disease that results when mice are orally infected with *Salmonella enterica* (10). Such resistance of TLR-deficient mice to pathogens bearing their cognate ligands has been attributed to a potentially harmful role for TLR-mediated production of proinflammatory cytokines and/or a role for TLR signaling (direct or indirect) in promoting dissemination of the pathogen. However, in the case of mice lacking TLR5, we hypothesized that their resistance to *Salmonella* may be more attributable to the loss of TLR5’s effect on their basal phenotype rather than to TLR5 playing a role in the detection of *Salmonella* flagellin during the infection per se. Specifically, we

recently observed that about 40% of TLR5-deficient (TLR5KO) mice exhibited some degree of gross and/or histopathologic spontaneous colitis (11). Although many TLR5KO mice had no histological evidence of colitis, such noncolitic TLR5KO mice had elevated levels of fecal and colonic bacteria and, moreover, exhibited TLR4-mediated elevation of proinflammatory gene expression. We thus hypothesized that these basal differences in the microbiota and/or their concomitant effects on basal gene expression play a role in the outcome of TLR5KO mice challenged with *Salmonella*. Therefore, we (i) examined the TLR5KO phenotype in a *Salmonella* gastroenteritis model in which microbiota is first decimated via antibiotic treatment and (ii) investigated whether the recently reported TLR5KO mouse resistance to *Salmonella*-induced typhoid-like illness might result from the basal phenotype of the mice.

## MATERIALS AND METHODS

**Mice.** TLR5KO mice and wild-type (WT) C57BL/6 littermates at N6 backcrossing were generated and comaintained as recently described (11). MyD88KO mice (backcrossed more than 10 generations) were compared directly to true C57BL/6 mice. All studies used mice between 6 and 10 weeks of age and did not observe age-related differences within this age window. All animal experiments were approved by the Emory University ethical committee.

***Salmonella* infections.** All infections described herein used *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain SL3201 or the previously described isogenic *fljC fljB* mutant (8). Gastroenteritis was induced by *Salmonella* by a method adopted from Barthel et al. (2) as recently described (12). Briefly, seemingly healthy mice (TLR5KO, WT, MyD88, or C57BL/6) were given streptomycin (7.5 mg/mouse in 0.1 ml sterile water; Sigma) by gavage and, after 24 h,

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† Supplemental material for this article may be found at <http://iai.asm.org/>.

∇ Published ahead of print on 14 January 2008.

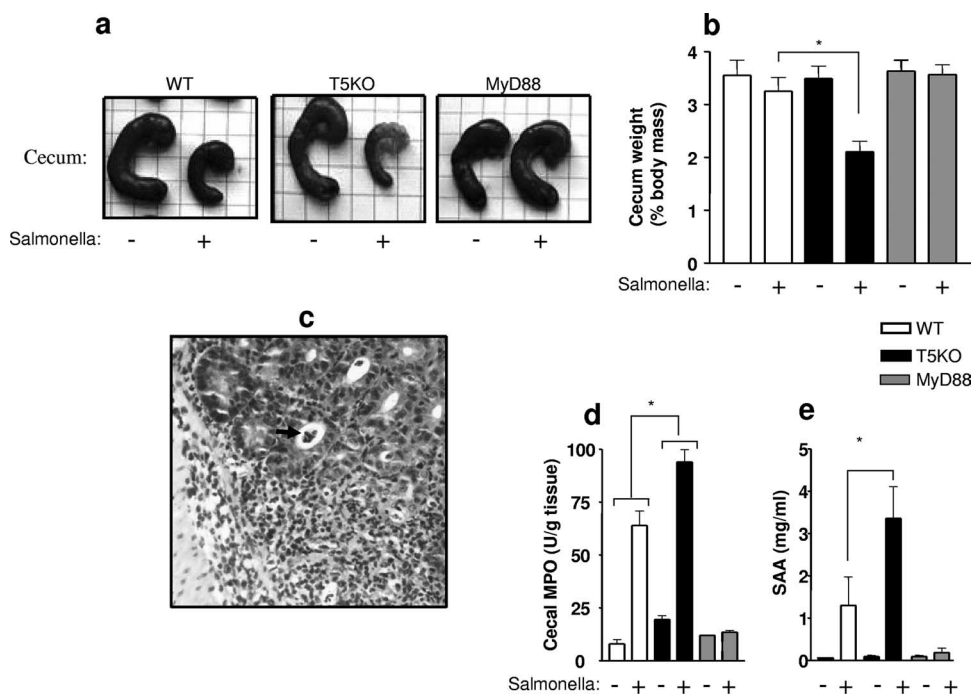


FIG. 1. TLR5KO mice develop exacerbated *Salmonella*-induced gastroenteritis that is driven by a MyD88-dependent pathway. Mice (WT, TLR5KO with low SAA levels, or MyD88KO; six mice per condition) were treated with streptomycin for 24 h and then administered  $10^8$  CFU *Salmonella* serovar Typhimurium by oral gavage. The mice were euthanized 48 h later. (a) Representative gross cecal pathology. (b) Measure of cecal weight as a quantitative assessment of gross cecal pathology. \*, statistically significant difference ( $P < 0.05$ ) between the WT and TLR5KO mice receiving *Salmonella*. (c) Representative image showing the severe inflammatory pathology of TLR5KO mice. The arrow indicates a crypt abscess. (d) Measure of levels of cecal MPO as quantitative assessments of the inflammatory pathology induced in this disease model. \*, statistically significant difference ( $P < 0.05$ ) between the WT and TLR5KO mice in the levels of *Salmonella*-induced MPO (i.e., after subtracted basal values). (e) Measure of SAA levels as a general indicator of health stress. \*, statistically significant difference ( $P < 0.05$ ) between WT and TLR5KO mice that received *Salmonella*.

were orally challenged with  $10^8$  CFU *Salmonella* serovar Typhimurium (SL3201). Cecal pathology was assayed 48 h later. Standard *Salmonella* serovar Typhimurium infections (i.e., without streptomycin) were performed on overnight-fasted male mice. Body weights were monitored every day, and animals which lost more than 25% of their body weight and were moribund were considered dead.

**Small intestinal mRNA/protein assays.** The small intestines of TLR5KO mice and WT littermates were assayed via procedures for the analysis of the colon that have been recently described (11). For mRNA-based assays (microarray and real-time PCR), mRNA isolated from whole small intestine was used. Protein assays used supernatants from 24-h-cultured 1-cm segments taken 10 cm proximal to the cecum. Antibodies to Ang4 and RegIII $\gamma$  were kindly provided by Lora Hooper (University of Texas Southwest Medical Center). Serum immunoglobulin G (IgG) was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (9). Serum IgA was measured by ELISA using IgA-specific antibodies from Southern Biotech (Birmingham, AL). For fecal Ig measurements, freshly isolated feces were suspended at 100 mg/ml in phosphate-buffered saline and assayed like serum Ig.

**Statistics.** Statistical significance was assessed by Student's *t* test, with *P* values less than 0.05 considered significant.

## RESULTS

Approximately 40% of TLR5KO mice exhibit detectable histopathologic evidence of spontaneous colitis, the severity of which corresponds to their level of the general inflammatory marker serum amyloid A (SAA) (11). TLR5KO mice with SAA concentrations below 50  $\mu$ g/ml lack significant histological evidence of colitis and thus were utilized herein to examine how TLR5KO mice fared when challenged with *Salmonella*.

**Loss of TLR5 results in exacerbated gut pathology in *Salmonella* gastroenteritis.** While oral infection of mice with *Salmonella* serovar Typhimurium has traditionally been used as a model of typhoid fever, Barthel and colleagues have characterized a model of *Salmonella*-induced gastroenteritis wherein mice develop robust intestinal inflammation when pretreated for 24 h with streptomycin and then colonized with *Salmonella* serovar Typhimurium (2). The presumed role of streptomycin in this model is that it increases colonization via ablation of the microbiota prior to colonization. In setting up this model, we verified that the streptomycin treatment indeed resulted in a marked reduction (over 3 logs) in the microbiota, at least in terms of fecal bacteria that could be grown in ambient (aerobic) conditions on nonselective agar. Flagellin-induced signaling is thought to be protective in this model in that flagellin-deficient *Salmonella* serovar Typhimurium causes greater pathology (12), although it is not known whether such an effect is mediated by TLR5 and/or one of the recently identified intracellular flagellin receptors (3–5, 7). As shown in Fig. 1, TLR5KO mice exhibited more severe gut pathology in this model (measured 48 h postinfection) than did their WT littermates, thus “phenocopying” the effect that deleting *Salmonella* serovar Typhimurium's flagellin genes has on disease observed in WT mice. Specifically, TLR5KO mice exhibited greater gross cecal pathology and concomitant loss of cecal weight and more severe inflammatory pathology than did their WT litter-

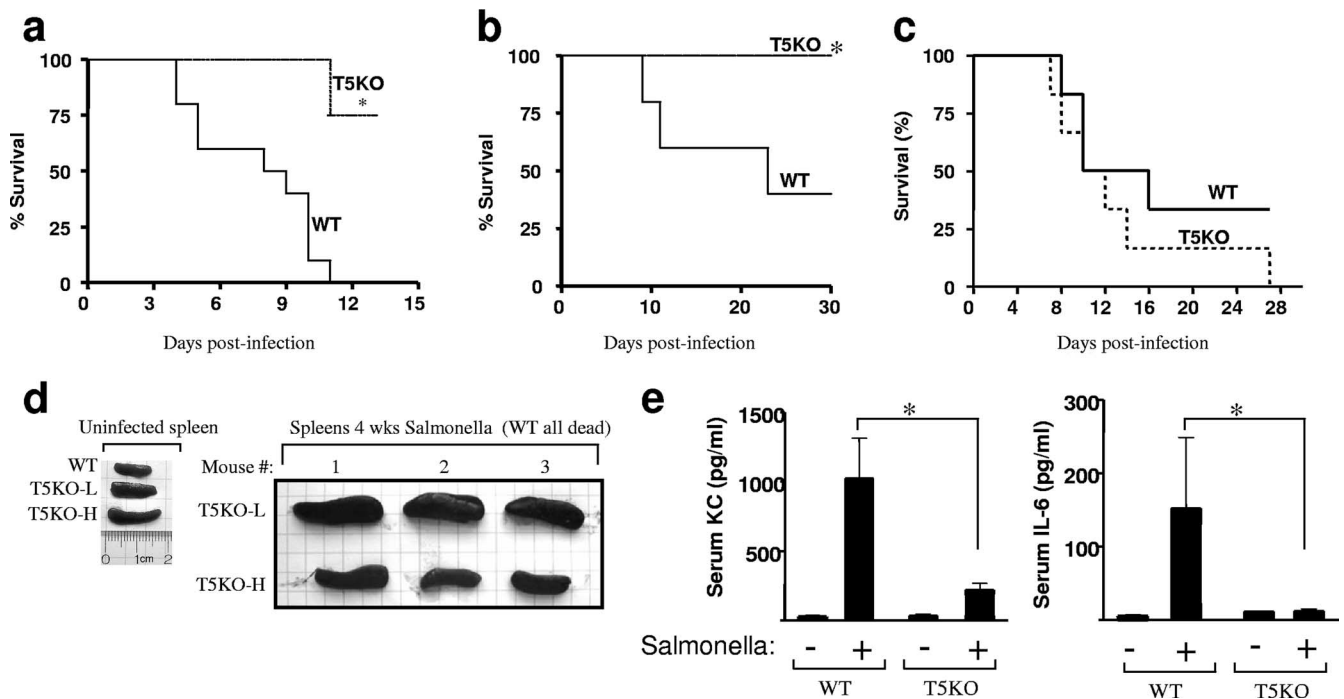


FIG. 2. Resistance of TLR5KO mice to *Salmonella*-induced typhoid-like illness is not specific to flagellated *Salmonella* bacteria and is ablated by antibiotic pretreatment. (a to c) TLR5KO mice (low SAA level except where indicated in panel d) and WT littermates were administered *Salmonella* serovar Typhimurium (SL3201) by oral gavage as follows, and survival was monitored:  $10^7$  CFU WT *Salmonella* (a),  $10^6$  CFU flagellin-deficient *Salmonella* serovar Typhimurium (SL3201 *fljC fljB*) (b), and 24 h of streptomycin pretreatment followed by  $10^7$  WT *Salmonella* serovar Typhimurium (SL3201) (c). Data shown are results from experiments using 6 to 10 mice for each condition. Each experiment was performed at least three times, and similar results were obtained each time. (d) Colitic and noncolitic TLR5KO mice (T5KO-H and T5KO-L, respectively) and WT littermates were colonized with  $10^6$  CFU aflagellate *Salmonella* serovar Typhimurium as described above. Spleens from knockout mice were examined 4 weeks postinfection. (e) Serum keratinocyte-derived chemokine (KC) and IL-6 levels in mice ( $n = 5$ ) that had been infected with  $10^7$  CFU WT *Salmonella* serovar Typhimurium (SL3201) and bled 5 h later; serum cytokine levels were quantitated by ELISA. Data are the means  $\pm$  standard errors of the means. \*, significant difference from WT mice ( $P < 0.05$ ).

mates, as indicated by both the presence of crypt abscesses and larger increases in cecal myeloperoxidase (MPO) and the general inflammatory marker SAA. This result suggests that TLR5-mediated innate immunity protects the gut from this gastroenteritis-causing pathogen. In contrast, mice lacking the general TLR-signaling adaptor MyD88, thus being broadly deficient in signaling by TLR5 and other TLR, showed no evidence of gut pathology (gross, microscopic, or biochemical) in this model. Thus, the loss of TLR5 signaling results in greater gut inflammatory pathology in response to *Salmonella* that is likely dependent upon signaling by other TLR.

**TLR5KO resistance to *Salmonella*-induced typhoid-like illness is not specific for flagellated *Salmonella* serovar Typhimurium.** If mice are not pretreated with antibiotics, oral administration of *Salmonella* serovar Typhimurium does not typically induce robust gut inflammation but rather disseminates the bacteria systemically and results in a disease state reminiscent of typhoid fever. Recent work by Uematsu et al. found that TLR5KO mice were resistant to *Salmonella* serovar Typhimurium administered in this manner (10) based upon their increased survival and reduced bacterial loads in the liver and spleen. The resistance was attributed to the loss of dendritic cell-mediated trafficking of the pathogen that may result when *Salmonella* flagellin ligates TLR5 to intestinal dendritic cells. However, our recent description of the basal phenotype of

TLR5KO (11) suggests an alternate mechanism of TLR5KO mouse resistance to this pathogen. Specifically, we observed that even noncolitic TLR5KO mice exhibited alterations in levels of their microbiota that were associated with an elevated expression of a number of genes with antimicrobial function. Thus, we hypothesized that these basal alterations in TLR5KO mice might render the gut generally resistant to orally administered pathogens via competitive exclusion and/or the increased activation of antibacterial/immune activating gene expression that is induced by the increased bacterial load.

To test the hypothesis that the resistance of TLR5KO mice to *Salmonella*-induced mortality might be nonspecific and ultimately attributable to altered levels of microbiota, we examined (i) whether the resistance was specific for flagellated *Salmonella* and (ii) whether the relative resistance was reduced by treatment with antibiotics. We observed that, compared to WT littermates, TLR5KO mice were relatively resistant to both flagellated and aflagellate *Salmonella* (Fig. 2a and b). Such resistance correlated with reduced bacterial loads in the liver/spleen (10; our data not shown). Furthermore, we observed that the relative resistance of TLR5KO mice to WT *Salmonella* was eliminated by pretreating the mice with streptomycin to decimate the microbiota (Fig. 2c). These results suggest that the resistance of TLR5KO mice to orally administered *Salmonella* serovar Typhimurium is more generally attributable to



the basal state of the intestine than to a role for TLR5 in *Salmonella* pathogenesis per se. To further examine the role of the basal state of the gut in determining the outcome of *Salmonella* infection, we next examined the outcome of *Salmonella* infection in TLR5KO mice with elevated basal levels of SAA. Such mice (referred to as T5KO-H mice) have robust spontaneous colitis and have elevations in antibacterial/immune-promoting gene expression that are considerably greater than those of noncolitic TLR5KO mice with low SAA levels (T5KO-L mice). As expected, all WT littermates died within 2 weeks of infection, while mice lacking TLR5 (T5KO-L and T5KO-H mice) were alive at 4 weeks, at which time some of them were euthanized and their organs were examined. TLR5KO mice have basally enlarged spleens, the extent of which is greater in T5KO-H mice (Fig. 2d) (11); however, at 4 weeks postadministration of *Salmonella*, the T5KO-L mice exhibited severe splenomegaly, while the spleens of the T5KO-H mice were only modestly enlarged beyond that seen in uninfected T5KO-H mice. While at this time point the mice had cleared the bacteria, the greater *Salmonella*-induced splenomegaly in the T5KO-L mice likely reflects a greater peak bacterial load in the spleens of the T5KO-L mice. The fact that the colitic subset of TLR5KO mice, which has a more pronounced basal phenotype and greater elevations in antibacterial/proinflammatory gene expression, exhibits such relatively reduced pathological change further supports the notion that the resistance of TLR5KO mice to oral *Salmonella* challenge results from their basal gut phenotype, rather than reflecting a role for TLR5 activation in mediating *Salmonella* dissemination. While such basal differences make it difficult to determine the physiological role of TLR5 in *Salmonella* infection, the fact that the WT littermates, but not the TLR5KO mice, made a rapid serum cytokine response to oral colonization with flagellated *Salmonella* serovar Typhimurium (Fig. 2e), but not with an aflagellate mutant (12), supports a major role for TLR5 in the initial recognition of this pathogen in the gut.

**TLR5KO mice exhibit elevated levels of innate and adaptive gut mucosal immunity.** The extensive list of host defense genes whose expression is elevated in the colon of TLR5KO mice suggests that various specific mediators may contribute to the resistance of these mice to orally administered *Salmonella* serovar Typhimurium. However, a significant portion of this pathogen's dissemination may, in fact, occur via the small intestine, which appears normal in TLR5KO mice (11). Moreover, our assessment of the small intestinal microbiota by both a culture-based method and Giemsa staining did not reveal any differences in these parameters (data not shown). Therefore, we examined via microarray analysis whether some of the elevations in host defense gene expression that we recently observed in the TLR5KO colon extended to the small intestine. The overall level of altered gene expression in the TLR5KO small intestine was less than that seen in the colon (see Table S1 in the supplemental material). Nonetheless, a number of host defense genes were observed to have elevated expression in the TLR5KO small intestine. Some of these genes, e.g., the MMP-9 gene, also had elevated expression in the colon, whereas others, e.g., the Ang4 and RegIII $\gamma$  genes, lacked significant elevation in the colon (11). We verified elevated protein expression for several of these genes, including the Ang4, RegIII $\gamma$ , and MMP9 genes (Fig. 3a). In contrast to colonic

supernatants, small intestinal supernatants from WT and TLR5KO mice lacked detectable keratinocyte-derived chemokine, interleukin 6 (IL-6), tumor necrosis factor alpha, and IL-1 $\beta$ . However, IL-18, which was not substantially elevated in the colon (11), was detectable, and in accordance with microarray results, levels of IL-18 protein were markedly elevated in TLR5KO mice (Fig. 3b). IL-18 is known to play a key role in innate immunity to *Salmonella* dissemination and thus may play a role in protecting TLR5KO mice from this pathogen (6). We also examined the small intestinal expression of lumican, which has recently been appreciated as playing a key role in innate immune recognition of lipopolysaccharide (15) and is highly upregulated in the TLR5KO colon (18.6- and 26.7-fold in T5KO-L and T5KO-H mice, respectively, as determined by microarray analysis). Small intestinal expression of lumican was highly elevated by quantitative reverse transcription-PCR (Fig. 3c). Another group of proteins whose altered mRNA expression in TLR5KO mice caught our attention was that of the polymeric IgA receptor and the "neonatal" IgG receptor FcRn, which are Ig transporters that transport IgA and IgG, respectively, to and from the intestinal lumen. The expression of these Ig transporters in the gut can protect mice from some enteric pathogens (14, 16). In accordance with the elevated intestinal expression of the polymeric IgA receptor (Fig. 3c) and FcRn (elevated 22.9-fold and 15.1-fold in T5KO-L and T5KO-H colons, respectively, as determined by microarray analysis), the TLR5KO mice exhibited elevated levels of both fecal IgG and IgA (Fig. 3d). IgG and IgA levels were also significantly elevated in TLR5KO serum. Such elevations in Ig concentration and transport may provide a degree of protection against a variety of microbes and/or represent increased immunosurveillance, thus allowing quicker induction of more specific pathways of adaptive immunity. We propose that these elevations in adaptive host defense pathways work in concert with the elevations in innate immunity to confer to TLR5KO mice elevated resistance to orally administered WT and aflagellate *Salmonella* serovar Typhimurium bacteria.

## DISCUSSION

Murine models of infection have proven to be powerful tools for studying mechanisms of microbial pathogenesis/host defense, especially when one is able to manipulate genes in both the pathogen and the host. Ideally, one observes that the deletion of a particular gene(s) in the pathogen and the gene(s) with which it interacts in the host produces similar phenotypes in the disease model and thus gives confidence to the conclusions drawn therefrom. This was the case in the study of the flagellin-TLR5 interaction in the streptomycin-*Salmonella* enteritis model, in which the deletion of TLR5 in the mouse "phenocopied" the result obtained when the flagellin genes were deleted from *Salmonella* serovar Typhimurium. The fact that both deleting TLR5 in the mouse and deleting *Salmonella*'s flagellin genes resulted in more severe intestinal pathology (12) allows us to conclude, with reasonable confidence, that the flagellin-TLR5 interaction protects the murine intestine against this pathogen. We speculate that the primary means by which the flagellin-TLR5 interaction protects the host in this model is by inducing expression of antibacterial proteins and immune-promoting cytokines that, directly and indirectly, re-

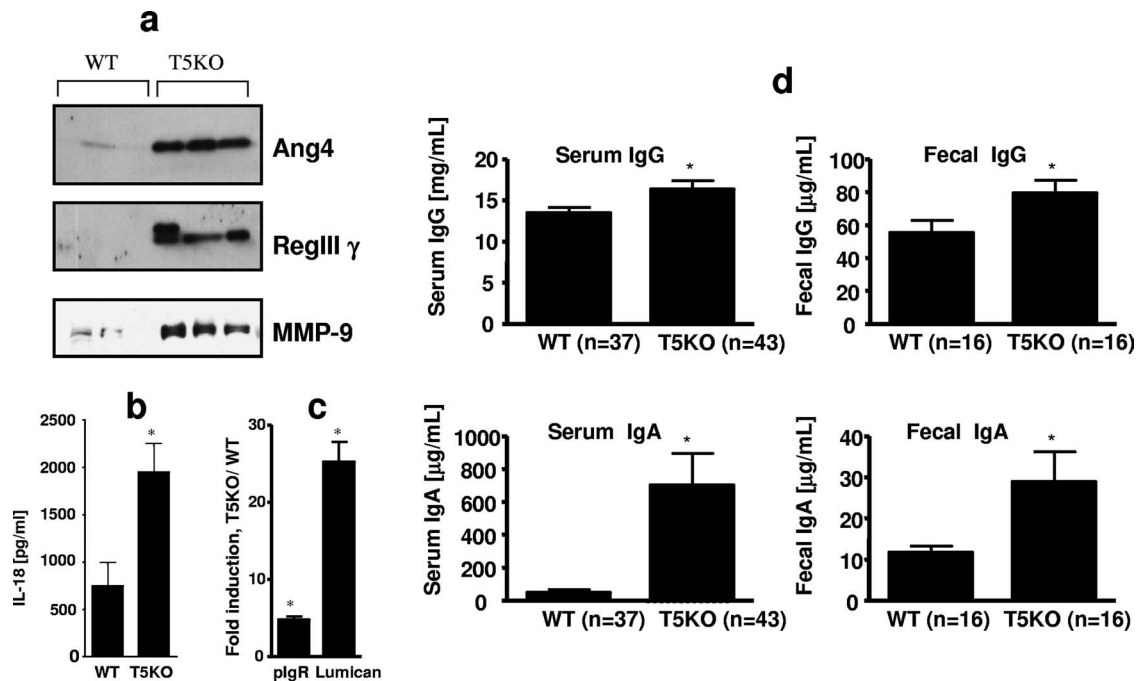


FIG. 3. TLR5KO mice have elevated expression of genes that mediate innate and adaptive immunity in the gut. (a and b) Small intestines from WT and T5KO-L mice were cultured ex vivo for 24 h in antibiotic-supplemented serum-free medium. Proteins released into the medium were assayed for angiogenin-4 (Ang4), RegIII $\gamma$ , and matrix metalloproteinase-9 (MMP-9) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblotting (a) or for IL-18 by ELISA (b). Results in panel a are from three individual mice and are representative of the results for six mice per condition. Results in panel b are means  $\pm$  standard errors of the means ( $n = 6$ ). (c) RNA levels from whole small intestine were assayed for levels of polymeric Ig receptor (pIgR) and lumican by quantitative reverse transcription-PCR. Results are means  $\pm$  standard errors of the means ( $n = 3$  mice). (d) Sera and suspensions of feces from TLR5KO mice or WT littermates were assayed for the total levels of IgG and IgA. Results are means  $\pm$  standard errors of the means ( $n = 16$  to 43), \*, significant difference from WT mice ( $P < 0.05$ ).

tard the bacteria. Additionally, ligation of TLR5 activates cytoprotective/antiapoptotic gene expression (17) that may also serve to protect the host intestine from the harmful affects of this pathogen.

We expected that the loss of TLR5 would also render mice more susceptible to *Salmonella*-induced typhoid-like illness that results when *Salmonella* disseminates to the liver/spleen. Such a result would have been analogous to recent observations that the dissemination of uropathogenic *Escherichia coli* from the urinary tract to the kidney is increased upon the loss of TLR5 (1). However, we and others observed that TLR5KO mice had increased resistance to *Salmonella* dissemination (10). This appears to contrast with the observations that the deletion of *Salmonella*'s flagellin genes increases virulence in mice (8), which compels us to give greater consideration to any potential effects that deleting such genes may have on various aspects of the pathogen or host. This led us to perform experiments that revealed that the resistance of TLR5KO mice to *Salmonella*-induced typhoid-like illness was not specific for flagellated *Salmonella* bacteria. Rather, such resistance correlated with alterations in their basal phenotype. Specifically, the small intestines and colons of TLR5KO mice exhibited elevations in basal levels of genes/proteins that mediate innate and adaptive immunity in the gut. Both the extent of such alterations and resistance to *Salmonella* dissemination were greater in mice that were overtly colitic (with high SAA levels), suggesting that the resistance may result from the elevated basal expression of host defense genes in the gut. The inability of

TLR5KO mice to control the microbiota likely underlies such alterations in host defense pathways. Such elevated antimicrobial gene expression is likely mediated, at least in part, by TLR4 in that alterations in gene expression are absent in TLR4/5 double knockout mice (11).

Another potential, and by no means mutually exclusive, explanation for the resistance of TLR5KO mice to orally administered *Salmonella* serovar Typhimurium is that the alterations in the microbiota prevent colonization via competitive exclusion. In support of this possibility, mucosal samples from T5KO-H mice have higher numbers of CFU (on nonselective agar in ambient conditions) than those from T5KO-L mice (11). While the lack of observed differences in the small intestinal microbiota argues against this possibility, the simple approaches used herein reflect populations of only a small portion of the microbiota (most are strict anaerobes and hard to culture) and cannot distinguish between different species of bacteria. Thus, non-culture-based methods are needed to assess the microbiota of TLR5KO mice to better understand its potential role in the TLR5KO phenotype. While the precise composition of the alterations in TLR5KO mouse microbiota are not yet defined, it should be noted that TLR5KO mice do not contain pathogenic bacteria per se, in that WT littermates that begin life with the same microbiota do not show any indications of disease (11). Thus, any resistance to infection they may develop as a result of changes in their microbiota would likely result from relative changes from within their

microbiota and not from the absolute presence or absence of a particular species.

If the resistance of TLR5KO mice to *Salmonella*-induced typhoid-like illness results from altered basal gene expression, it may not result from the induction of a single gene but rather from the combined actions of multiple host defense genes. The fact that such elevations in TLR5KO gene expression are associated with spontaneous colitis demonstrates the potential danger to the host of having these pathways continuously activated. However, it also suggests that it may be possible to therapeutically boost innate immunity temporarily as a potential means of providing nonspecific protection against a variety of pathogens.

#### ACKNOWLEDGMENTS

This work was supported by NIH grant DK061417 to A.T.G. M.V.-K. is a recipient of a research fellowship from the Crohn's and Colitis Foundation of America (CCFA). We acknowledge NIH Digestive Disease Research and Development Center (DDRDC) grant DK064399 to Emory University.

We thank Daniel Moore III for technical assistance.

We declare that we have no competing financial interests.

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Editor: A. J. Bäumlér