Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice

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The intestinal microbiota contributes to the development of the immune system, and conversely, the immune system influences the composition of the microbiota. Toll-like receptors (TLRs) in the gut recognize bacterial ligands. Although TLR signaling represents a major arm of the innate immune system, the extent to which TLRs influence the composition of the intestinal microbiota remains unclear. We performed deep 16S ribosomal RNA sequencing to characterize the complex bacterial populations inhabiting the ileum and cecum of TLR− and MyD88−deficient mice. The microbiota of MyD88− and TLR−deficient mouse colonies differed markedly, with each colony harboring distinct and distinguishable bacterial populations in the small and large intestine. Comparison of MyD88−, TLR2−, TLR4−, TLR5−, and TLR9−deficient mice and their respective wild-type (WT) littermates demonstrated that the impact of TLR deficiency on the composition of the intestinal microbiota is minimal under homeostatic conditions and after recovery from antibiotic treatment. Thus, differences between TLR−deficient mouse colonies reflected long-term divergence of the microbiota after extended husbandry in isolation from each other. Long-term breeding of isolated mouse colonies results in changes of the intestinal microbiota that are communicated to offspring by maternal transmission, which account for marked compositional differences between WT and mutant mouse strains.

Complex microbial populations inhabit the mammalian gastrointestinal tract and are referred to as the microbiota. Next generation DNA sequencing platforms have been used to sequence 16S ribosomal RNA (rRNA) genes amplified from complex bacterial populations and have enabled investigation of the gut microbiota, revealing that thousands of different bacterial species contribute to the commensal community (Dethlefsen and Relman, 2011). Most of the bacterial taxa in the gut belong to the Firmicutes and Bacteroidetes phyla, whereas bacteria belonging to the Proteobacteria, Actinobacteria, Verrucomicrobia, or Fusobacteria phyla form minority populations (Eckburg et al., 2005). The bacterial communities inhabiting the small and large intestine are distinct (Gamer et al., 2009). Furthermore, microbial populations in close proximity to the intestinal epithelium differ from those inhabiting the intestinal lumen (Hill et al., 2010).

The intestinal microbiota provides many benefits for its host. Intestinal bacteria contribute to the gut’s primary function of absorbing nutrients by producing enzymes that break down otherwise indigestible compounds (Hooper et al., 2002). Intestinal bacteria also contribute to the development of the immune system (Hill and Artis, 2010) and enhance resistance against infection by pathogenic bacteria (Vollaard and Clasener, 1994). The intestinal microbiota promotes angiogenesis in the intestine (Stappenbeck et al., 2002) and provides protection against intestinal epithelial injury (Rakoff-Nahoum et al., 2004). Changes in the composition of the microbiota...
can contribute to diseases, including obesity (Ley et al., 2005), diabetes (Wen et al., 2008), autoimmunity (Wu et al., 2010), inflammatory bowel disease (Elinav et al., 2011), and infectious diseases (Donskey et al., 2000).

The composition and structure of commensal bacterial populations inhabiting the intestine can be altered by exogenous factors. Maternal transmission represents the earliest and arguably most important factor determining the composition of the microbiota (Spor et al., 2011). It has been shown that the microbiota of littermate mice is more similar than the microbiota of genetically identical mice that differ in maternal origin (Ley et al., 2005). In humans, the microbiota of siblings is more similar than the microbiota of unrelated individuals, which can differ markedly (Turnbaugh et al., 2009a). Diet is another environmental factor that influences composition of the gut’s microbial populations. A study using germ-free mice that have been colonized with microbiota isolated from humans demonstrated that switching from a low-fat, plant polysaccharide–rich diet to a high-fat, high-sugar diet changed the structure of the microbiota within one day (Turnbaugh et al., 2009b). We and others have shown that antibiotic treatment can dramatically alter intestinal microbial diversity (Dethlefsen et al., 2008; Sekirov et al., 2008; Antonopoulos et al., 2009; Hill et al., 2010; Ubeda et al., 2010; Dethlefsen and Relman, 2011). Some changes in the microbiota after antibiotic administration persist for prolonged periods of time (Jernberg et al., 2007, 2010; Ubeda et al., 2010; Dethlefsen and Relman, 2011; Buffie et al., 2012).

In addition to exogenous factors, the immune system also shapes the composition of the intestinal microbiota. Mice deficient in IgA become densely colonized by segmented filamentous bacteria (SFB; Suzuki et al., 2004). Mice with double Rag and T-bet deficiency became colonized with a microbiota that promoted intestinal inflammation and that, upon transfer into WT mice, induced colitis (Garrett et al., 2007, 2010; Ubeda et al., 2010; Dethlefsen and Relman, 2011; Buffie et al., 2012).

To investigate the impact of TLR signaling on the composition of the intestinal microbiota, we crossed C57BL/6J female mice that were heterozygous for deletion of the myeloid differentiation factor MyD88 gene (MyD88<+/−>) with C57BL/6J MyD88<+/−> male mice and determined the genotype of pups. MyD88<+/−> mice were paired with either MyD88<+/> or MyD88<+/−> mice from the same litter and were separated at the time of weaning (21 d after birth) into individual, autoclaved cages. Mice were euthanized 5–7 wk after weaning, and the composition of the microbiota colonizing the ileum epithelium and lumen and cecum lumen was determined by sequencing of PCR–amplified bacterial 16S rDNA using the Roche/454 platform. The same approach was used to analyze the impact of TLR2, TLR4, TLR5, and TLR9 expression on the intestinal microbiota. For each mutant strain, our analyses included 5–7 KO mice and 5–7 WT (either +/+ or +/−) littermate controls. A total of 586,351 sequences passed our quality criteria (see Materials and methods) and were used in this analysis. For our analysis of the impact of MyD88 deficiency on the cecal microbiota, we obtained a mean of >10,000 sequences per sample, whereas for the other comparisons, we obtained a mean of >2,600 sequences.

We first characterized the effects of TLR signaling on the microbiota composition by quantifying similarities based on phylogenetic distances using UniFrac (Lozupone and Knight, 2005). We used the unweighted UniFrac metric to tabulate the presence or absence (but not prevalence) of different bacterial taxa and principal coordinate analysis (PCoA) to cluster communities along orthogonal axes of maximal variance. To analyze whether different TLRs make distinct contributions to the composition of the microbiota, we used PCoA to compare all the cecal samples obtained from the different mutant mouse colonies and their respective WT
Jackson Laboratory C57BL/6 mice. It is likely, therefore, that the microbiota differences between our MyD88- and TLR-deficient colonies result from prolonged separate breeding and initial colonization with a distinct flora upon transfer to our facility.

To determine whether maternal transmission of microbiota to sequential litters is similar or variable over time, we separately analyzed microbial populations from distinct litters. Cecal samples from two different litters raised by the same MyD88 heterozygous parents were distinct, as demonstrated by PCoA (Fig. 1 A, MyD88 panel). However, in both litters, MyD88 KO and WT littermates clustered together, demonstrating that changes in the microbiota between litters were more pronounced than changes resulting from defective MyD88 signaling. Similar results were obtained when mice from the TLR2 colony were analyzed. In this case, KO and littermate WT mice were obtained from three different heterozygous TLR2-deficient parents. Although KO and WT mice from the same litter that had a different genotype, or (c) all other mice within that colony belonging to a different litter were calculated and plotted. A higher UniFrac distance denotes greater dissimilarity between two microbial communities. Error bars indicate ±SEM. Asterisks denote statistically significant differences in UniFrac distances between groups (Student’s t test: *, P < 0.05; ***, P < 0.001).

controls (Fig. 1 A, all colonies). Although the first two principal coordinates of analysis (PC1 and PC2) separated mice belonging to different mutant colonies, there was no separation between WT and KO mice belonging to the same litter (Fig. 1 A, closed or open circles). For example, the microbiota of WT littermates from the MyD88-deficient breeding colony clustered tightly with MyD88 KO mice but not WT mice from TLR-deficient colonies, indicating that the composition of their microbiota was largely determined by maternal transmission and not the presence or absence of TLR signaling in their host. The microbiota derived from WT and KO mice of each TLR-deficient colony also clustered together (Fig. 1 A), although for the TLR2-deficient colony two different litters clustered separately. Comparison of cecal microbiota using the weighted UniFrac analysis, which takes into account the frequency of different bacterial taxa demonstrated clustering of microbial populations derived from TLR5−, TLR4−, and TLR2-deficient mouse colonies by PC1 and PC2 (not depicted). Similar to our analysis of the cecal microbiota, samples isolated from the ileal epithelium or ileal lumen of KO mice and WT littermate controls were not distinguishable by PCoA using unweighted UniFrac analysis. However, PC1 and PC2 separated the microbiota from mice derived from TLR2− and TLR4-deficient mice from the other mouse colonies (not depicted). The colonies of MyD88−/−, TLR2−/−, TLR4−/−, TLR5−/−, and TLR9−/− mice that were used to compare KO with WT littermate controls had been bred and maintained separately for at least 5 yr after initial transfer to our facility and further backcrossing to Jackson Laboratory C57BL/6 mice. It is likely, therefore, that the microbiota differences between our MyD88−/− and TLR-deficient colonies result from prolonged separate breeding and initial colonization with a distinct flora upon transfer to our facility.

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Although the preceding results indicate that TLR signaling does not alter the microbiota, it remained possible that specific bacterial taxa might be affected by defects in TLR signaling. To address this, we determined the relative representation of different bacterial taxa in intestinal samples. In most cases, classification could be assigned to the genus level. Fig. 3 shows the relative proportions of the most abundant taxa identified in MyD88−/−, TLR2−/−, TLR4−/−, TLR5−/−, and TLR9−/− mice and their respective WT littermates. As previously shown (Ubeda et al., 2010), most bacteria belong to the Bacteroidetes and Firmicutes phyla and the composition of the microbiota from ileum and cecum differ. The ileum epithelium of some mice in all colonies was dominated by SFB. The level of SFB colonization did not differ between KO and WT littermates, with the exception of litter E of the TLR4 colony, where SFB dominated the microbiota of TLR4−/− mice but not their respective WT littermates (not depicted).

To identify bacterial taxa that are significantly affected by TLR signaling, we used the Student’s t test to compare the relative amount of specific taxa colonizing KO and littermate WT mice. Taxa of low abundance (mean in both groups less than five counts) were not included in this analysis. To avoid false positives as the result of multiple comparisons, the Benjamini and Hochberg false discovery rate (FDR) was applied to those taxa that differed significantly (P < 0.05). Although a p-value <0.05 was obtained for several bacterial taxa, none passed the FDR criteria (≤0.1; Fig. 4). Similar results were obtained with a paired Student’s t test in which samples were paired by litter origin or when a nonparametric test (Wilcoxon test) was used to calculate statistically significant changes (not depicted).

No differences were observed between KO and WT mice when analysis was performed at the operational taxonomic unit (OTU) level (≥97% identity). In contrast, several taxa were found to be significantly different when intestinal samples from different litters were analyzed. UniFrac distances for each mouse in a specific colony versus (a) all the other mice from the same litter with the same genotype, (b) all other mice from the same litter that had a different genotype, or (c) all other mice within that colony belonging to a different litter. A lower UniFrac distance denotes greater similarity between two microbial communities. Thus, if the microbiota of KO mice differed from that of WT littermates, a higher UniFrac distance would be expected between mice with different genotypes than between mice with the same genotype. As shown in Fig. 1 B for cecal samples, UniFrac distances between mice of a different genotype were not higher than UniFrac distances between mice of the same genotype. However, higher UniFrac distances were observed between mice from different litters. Similar results were obtained with ileal samples from mice belonging to different litters (not depicted), suggesting that maternal origin has a greater impact on the composition of the microbiota than TLR signaling. To demonstrate that maternal origin plays a major role shaping the composition of the intestinal microbiota, we applied the UPGMA (unweighted pair group method with arithmetic mean) clustering algorithm to the unweighted UniFrac distances of all cecal samples. The UPGMA algorithm generates a tree in which samples with greater microbiota similarity cluster together. As shown in Fig. 2, mice with the same maternal origin, including different litters from the same mother, cluster together. In contrast, clustering by mouse genotype was much more limited, indicating that maternal origin is the major force driving the differences observed in the different mouse colonies.

**Figure 2.** Maternal effect on the microbiota composition. Cecal microbiota samples were clustered by similarity using the UPGMA clustering algorithm on the unweighted UniFrac distance matrix. Colors indicate the mouse maternal origin. Samples were named using the following four-label code: (1) mouse colony (M, MyD88; T2, TLR2; T4, TLR4; T5, TLR5; and T9, TLR9), (2) mouse family identified with a letter, with consecutive litters from the same mother differentiated with a number, (3) mouse genotype (H, heterozygous; K, KO; and W, WT), and (4) mouse ID number. Samples are clustered by maternal origin, as indicated with labels outside the circle tree. Consecutive litters from the same mother (distinguished by numbers) clustered together, indicating the major role of maternal origin on the microbiota composition from TLR-deficient colonies. Each mouse colony contains five to seven KO and five to seven WT/Het mice.
were found between KO and WT mice (not depicted). Similar results were obtained when we estimated species microbial richness using the Chao Index (not depicted).

Contribution of TLR signaling to changes in the intestinal microbiota after antibiotic treatment

We and others have shown that antibiotic treatment produces dramatic changes in the composition of the intestinal microbiota (Ubeda et al., 2010). To determine whether TLR signaling influences the recovery of microbial populations after antibiotic treatment, we treated 8–10-wk-old MyD88\(^{-/-}\), TLR2\(^{-/-}\), TLR4\(^{-/-}\), TLR5\(^{-/-}\), or TLR9\(^{-/-}\) mice and their respective WT littermates with oral vancomycin for 7 d and monitored their microbiota during and after treatment. We previously demonstrated that vancomycin induces dramatic changes in the intestinal microbiota that persist after cessation of treatment (Ubeda et al., 2010). To follow microbial changes in individual mice, we obtained fecal pellets before antibiotic treatment, after 7 d of vancomycin treatment, and 2 and 4 wk after stopping treatment, at which time mice were sacrificed and the luminal contents of cecum and ileum and the ileum epithelium were collected for microbiota analysis. All mice were individually housed after weaning, and a total of 1,354,358 sequences that passed quality criteria (mean/sample = 3,455) were used in this analysis. Figure 5 shows the relative amount of the most abundant taxa identified in fecal samples from MyD88\(^{-/-}\), TLR2\(^{-/-}\), TLR4\(^{-/-}\), TLR5\(^{-/-}\), and TLR9 KO mice.

To determine whether TLR signaling has an impact on the diversity of the intestinal microbiota, the Shannon diversity index was calculated for each intestinal sample. Student’s \(t\) test (\(P < 0.05\)) was used to determine whether the microbial diversity in KO mice was statistically different from that of WT littermates. No differences in the Shannon diversity index were found between KO and WT mice (not depicted). Similar results were obtained when we estimated species microbial richness using the Chao Index (not depicted).
TLR5−/−, and TLR9−/− mice and their respective WT littermates. As expected, the intestinal microbiota changed after 7 d of vancomycin treatment in all mouse colonies. Interestingly, although all mice, regardless of colony or genotype, developed some common changes (expansion of *Lactobacillus* and loss of *Barnesiella* and *Bacteroides*), individual colonies developed specific changes. For example, in TLR4- and TLR5-deficient colonies, expansion of *Parabacteroides* was observed, whereas expansion of *Prevotella* was observed in MyD88- and TLR5-deficient colonies. The changes in the microbiota of KO and WT littermates within a specific colony, however, were indistinguishable, suggesting that TLR signaling does not impact the recovery of microbial populations after antibiotic treatment. Consistent with previous results (Ubeda et al., 2010), antibiotic-treated KO or WT littermates that were individually housed did not recover their initial microbiota even 1 mo after stopping antibiotic treatment. To determine whether specific bacterial taxa significantly differed between KO and WT littermates, we used the Student’s *t* test. As noted in the comparison of microbial populations under homeostatic conditions, no bacterial taxa in KO or WT littermates significantly differed between KO and WT littermates, we used the Student’s *t* test. (P < 0.05), and TLR5−/− mice (Fig. 6 B).}

The composition of the microbiota before and after vancomycin treatment was compared in each KO colony by UniFrac and PCoA (Fig. 7). As can be seen for the MyD88 colony, PC1 separated untreated samples from samples obtained after 7 d of vancomycin treatment and samples obtained 2 and 4 wk after discontinuation. Thus, vancomycin induces prolonged changes to the microbiota. PC3 separated samples that were obtained after 7 d of vancomycin treatment from those obtained 2 or 4 wk after discontinuing vancomycin. This result indicates that after antibiotic withdrawal, the microbiota continues to evolve, with significant shifts in the composition over time. Importantly, the microbiota of KO and WT littermates changed similarly, suggesting that TLR signaling does not contribute to the expansion and contraction of distinct microbial populations after antibiotic treatment. PC2 separated samples by parental origin. In this case, two different litters were used to analyze differences between KO and WT littermates. Mice within the same litter had a more similar microbiota under steady-state conditions, and, despite the dramatic changes induced by vancomycin treatment, mice from different litters maintained a distinct microbiota. A similar pattern was observed when samples from TLR2−/-, TLR5−/-, and TLR9−/- deficient colonies were analyzed. We obtained similar results when the relative abundance of the different bacterial taxa was taken into account using the weighted UniFrac analysis (not depicted). Together, these results suggest that TLR signaling does not play a detectable role in shaping the intestinal microbiota during or after recovery.
from antibiotic treatment. Instead, our results suggest that maternal transmission of microbiota has by far the greatest influence on the composition of the intestinal microbiota, even in the aftermath of profound changes induced by antibiotic administration.

**DISCUSSION**

The intestinal microbiota has been implicated in the pathogenesis of diabetes, obesity, and inflammatory bowel disease. Deciphering the mechanisms that shape and maintain microbiota in the gut of mucosal surfaces may provide approaches to prevent or ameliorate a range of human diseases. We have used mice with defined genetic deletions and high-throughput sequencing techniques to determine whether TLR signaling influences the composition of the gut microbiota. We found significant differences in the microbiota of WT and MyD88−, TLR2−, TLR4−, TLR5−, or TLR9− deficient mouse colonies that had been housed and bred in isolation from each other for >5 yr. Careful comparison of WT and MyD88− or TLR3− deficient littermate control mice, in which offspring resulted from crosses of mice heterozygous for each deletion, however, demonstrates that deficiency in MyD88, TLR2, TLR4, TLR5, or TLR9 KO mice and WT/ Het littermate controls were treated with vancomycin (Vanco) for 7 d on their drinking water. Fecal samples were obtained before treatment, 1 wk after vancomycin treatment, and 2 and 4 wk after antibiotic withdrawal. Phylogenetic classification of 16S rDNA frequencies in fecal samples was obtained by high-throughput sequencing and mothur sequencing analysis. Each bar represents the mean of the microbiota composition from four to seven mice. The most predominant bacterial taxa are shown and labeled with different colors as indicated.

Figure 5. Impact of TLR signaling on the fecal microbiota composition after antibiotic treatment. MyD88, TLR2, TLR4, TLR5, or TLR9 KO mice and WT/ Het littermate controls were treated with vancomycin (Vanco) for 7 d on their drinking water. Fecal samples were obtained before treatment, 1 wk after vancomycin treatment, and 2 and 4 wk after antibiotic withdrawal. Phylogenetic classification of 16S rDNA frequencies in fecal samples was obtained by high-throughput sequencing and mothur sequencing analysis. Each bar represents the mean of the microbiota composition from four to seven mice. The most predominant bacterial taxa are shown and labeled with different colors as indicated.
that MyD88-mediated signals in the intestinal epithelium induce expression of the Reg3γ antimicrobial protein, which targets bacteria in an ~50-µm zone that separates the luminal contents and associated microbiota from the epithelial surface of the small intestine. MyD88- and Reg3γ-deficient mice both had increased bacterial densities in proximity to the intestinal epithelial surface, indicating that MyD88-driven Reg3γ expression promotes bacterial segregation from the intestinal epithelial surface. Consistent with the known antibacterial specificity of Reg3γ for Gram-positive bacteria, mice deficient for Reg3γ had increased numbers of Gram-positive bacteria (SFB and Eubacterium rectale) associated with the ileal epithelium. In contrast, however, the microbiota in the ileal lumen did not differ between Reg3γ−/− mice and their WT littermates, suggesting that the impact of TLR- and MyD88-induced Reg3γ on commensal bacteria is restricted to the region immediately adjacent to the intestinal epithelium. Our results demonstrate that MyD88 deficiency does not affect the relative proportions of different bacterial taxa in close proximity to the intestinal wall or within the intestinal lumen. MyD88 deficiency, in addition to inducing Reg3γ expression, also affects other antimicrobial effector pathways that may inhibit bacterial species with less specificity and thus leave the relative proportions of different taxa intact.

Figure 6. Impact of TLR signaling on the diversity and richness of the intestinal microbiota after antibiotic treatment. (A and B) Myd88, TLR2, TLR4, TLR5, or TLR9 KO mice and WT/Het littermate controls were treated with vancomycin (vanco) for 7 d on their drinking water. Fecal samples were obtained before treatment, 1 wk after vancomycin treatment, and 2 and 4 wk after antibiotic withdrawal. The Shannon diversity index and Chao richness index were obtained by 16s rDNA high-throughput sequencing and mothur analysis. Student’s t test was performed between groups of KO mice and their respective WT/Het controls to analyze the impact of TLR signaling on the overall microbial diversity and richness (n = 4–7 mice/genotype). Horizontal bars indicate the mean. The asterisk denotes significance (*, P < 0.05).
Vijay-Kumar et al. (2010) study could be detected in our mice colony, although their relative abundance was not significantly different in mice with different genotype. Thus, although the laboratory to obviate the potential impact of a deviant, institution-specific microbiota. Comparison of rederived TLR5 KO with WT littermate control mice revealed marked effects of TLR5 deficiency on the cecal microbiota. Furthermore, the microbiota shaped by TLR5 deficiency could, upon adoptive transfer into germ-free WT mice, induce many aspects of the metabolic syndrome in recipient mice. Thus, the microbiota that develops in TLR5-deficient mice can be distinct from the flora that develops in WT littermates and can lead to the development of metabolic syndrome. We did not observe differences between the microbiota of WT and TLR5 KO mice, and our colony of TLR5-deficient mice had no signs of metabolic syndrome such as obesity or mild intestinal inflammation. It is likely that these differences are impacted by husbandry conditions in different animal facilities. We compared the microbiota of our WT and TLR5-deficient mice with the microbiota of C57BL/6 mice that were recently purchased from the Jackson Laboratory and the microbiota of TLR5-deficient and WT control mice described by the Vijay-Kumar et al. (2010) study. PCoA of a UniFrac analysis revealed clear differences between these three mouse colonies (Fig. 8). As previously demonstrated, TLR5 deficiency impacted the microbiota composition of the mouse colony studied by Vijay-Kumar et al. (2010) but not our colony (Fig. 8). Most of the OTUs that were found to be significantly different between KO and WT mice from the Vijay-Kumar et al. (2010) study could be detected in our mice colony, although their relative abundance was not significantly different in mice with different genotype. Thus, although the
husbandry conditions and exposure to exogenous microbial populations in our animal facility do not lead to detectable differences between WT and TLR-deficient mouse strains, it is likely that conditions that are unique to specific animal facilities can accelerate the divergence of microbial populations colonizing different mouse colonies. One of these conditions might be colonization with SFB, which could influence the microbiota by enhancing Th17 differentiation. The extent to which SFB influences the microbiota in WT or TLR-deficient mice is unclear. Although our TLR-deficient mouse colonies are colonized with SFB, as determined by 16S sequencing of bacteria associated with ileal mucosa (their major habitat), it is unclear whether mice used in other studies were SFB colonized because microbiota analyses have been restricted to luminal samples. It is possible, therefore, that the presence or absence of SFB in TLR5-deficient mice might lead to divergent outcomes.

The observation that the microbiota remains stable in the absence of TLR signaling suggests that the complex bacterial populations inhabiting the gut establish an equilibrium that is at least in part self-sustaining. Experiments performed in other ecosystems, such as bacterial populations in soil, have shown that stability and resilience increase with the complexity of the ecosystem (Girvan et al., 2005). Thus, TLR signaling might have a greater effect on the stability and resilience of less complex microbial populations. Colonization of germ-free, TLR-deficient mice with a less complex microbiota such as ASF (alternated Schaedler flora) could address this question. In conclusion, although there is increasing evidence that microbiota changes associated with inflammatory diseases can and do occur in mouse strains with deficiencies in their innate immune signaling pathways (Elinav et al., 2011), it remains unclear how quickly these changes occur and to what extent these changes are institution specific.

MATERIALS AND METHODS

Mouse models and housing conditions. All mouse procedures were performed in accordance with institutional protocol guidelines at Memorial Sloan–Kettering Cancer Center (MSKCC). Mice were maintained according to National Institutes of Health Animal Care guidelines, under protocols approved by the MSKCC Institutional Animal Care Committee describing experiments specific to this study. To investigate the impact of TLR signaling on the composition of the intestinal microbiota, we crossed C57BL/6J WT mice with myeloid differentiation factor–deficient mice (MyD88−/−). Heterozygous (MyD88+/−) mice were crossed, and the genotype of pups was determined. MyD88−/− mice remained co-housed with WT littermates (either MyD88+/− or MyD88+/+) and were separated upon weaning (21 d after birth) into individual, autoclaved cages, containing irradiated food and acidified water. Mice were euthanized 5–7 wk after weaning for intestinal analyses. For experiments involving antibiotic treatment, mice were individually housed in the same conditions described above. Fresh stool pellets were obtained before mice were euthanized. Immediately after mice were euthanized, the contents of the cecum and the distal 3 cm of the small intestine (ileum), excluding the last 1 cm proximal to the cecum, were recovered by manual extrusion. The 3 cm of the ileum were flushed twice with PBS and saved for DNA extraction from the ileum epithelium. The samples were immediately frozen and stored at −80°C. Except for the ileum epithelium (see next paragraph), DNA was extracted using a phenol-chloroform extraction technique with mechanical disruption (bead beating) based on a previously described protocol (Turnbaugh et al., 2009a). In brief, a frozen aliquot (~100 mg) of each sample was suspended, while frozen, in a solution containing 500 μl of extraction buffer (200 mM Tris, pH 8.0/200 mM NaCl/20 mM EDTA), 210 μl of 20% SDS, 500 μl phenol/chloroform/isoamyl alcohol (24:24:1), and 500 μl of 0.1-mm-diameter zirconia/silica beads (BioSpec Products). Microbial cells were lysed by mechanical disruption with a bead beater (BioSpec Products) for 2 min, after which two rounds of phenol/chloroform/isoamyl alcohol extraction were performed. DNA was precipitated with ethanol and resuspended in 50 μl Tris-EDTA buffer with 100 μg/ml RNase. The isolated DNA was subjected to additional purification with QIAquick mini spin columns (QIAGEN).

Total genomic DNA was extracted from ileal wall samples using the QIAamp DNA isolation kit (QIAGEN) and a bead-beater method based on a previously described protocol (Bik et al., 2006). In brief, ileum wall fragments were lysed in 180 μl QIAamp ATL buffer and 20 μl proteinase K for 1 h at 56°C. Zirconia/silica beads (0.1-mm diameter) were added, and samples were homogenized in a bead beater (BioSpec Products) for 2 min and incubated for an additional hour at 56°C. 4 μl RNase A (100 mg/ml) and 200 μl AL buffer (QIAGEN) were added to the lysate, and samples were incubated for 30 min at 70°C. After the addition of 200 μl absolute ethanol, lysates were purified over a QIAamp column as specified by the manufacturer.

16S rDNA amplification and 454/pyrosequencing. For each sample, three replicate 25-μl PCRs were performed, each containing 50 ng of purified DNA, 0.2 mM dNTPs, 1.5 mM MgCl2, 1.25 U Platinum Taq DNA polymerase, 2.5 μl of 10× PCR buffer, and 0.2 μM of each primer designed to amplify the V1–V3 region as previously described (Bik et al., 2006). Two sequential litters obtained from the same MyD88−/− mouse colony, we collected samples from mice from different litters that were housed separately. Fresh stool pellets were obtained before mice were euthanized. Immediately after mice were euthanized, the contents of the cecum and the distal 3 cm of the small intestine (ileum), excluding the last 1 cm proximal to the cecum, were recovered by manual extrusion. The 3 cm of the ileum were flushed twice with PBS and saved for DNA extraction from the ileum epithelium. The samples were immediately frozen and stored at −80°C. Except for the ileum epithelium (see next paragraph), DNA was extracted using a phenol-chloroform extraction technique with mechanical disruption (bead beating) based on a previously described protocol (Turnbaugh et al., 2009a). In brief, a frozen aliquot (~100 mg) of each sample was suspended, while frozen, in a solution containing 500 μl of extraction buffer (200 mM Tris, pH 8.0/200 mM NaCl/20 mM EDTA), 210 μl of 20% SDS, 500 μl phenol/chloroform/isoamyl alcohol (24:24:1), and 500 μl of 0.1-mm-diameter zirconia/silica beads (BioSpec Products). Microbial cells were lysed by mechanical disruption with a bead beater (BioSpec Products) for 2 min, after which two rounds of phenol/chloroform/isoamyl alcohol extraction were performed. DNA was precipitated with ethanol and resuspended in 50 μl Tris-EDTA buffer with 100 μg/ml RNase. The isolated DNA was subjected to additional purification with QIAamp mini spin columns (QIAGEN).

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165 rDNA amplification and 454/pyrosequencing. For each sample, three replicate 25-μl PCRs were performed, each containing 50 ng of purified DNA, 0.2 mM dNTPs, 1.5 mM MgCl2, 1.25 U Platinum Taq DNA polymerase, 2.5 μl of 10× PCR buffer, and 0.2 μM of each primer designed to amplify the V1–V3 region as previously described (Bik et al., 2012); a modified primer 8F (5′-CCTATATCCCTGTGTTG-3′), a modified primer 8R (5′-GGCTCGGTCTCCGGCTGAGG-3′), and the modified primer 343R (5′-CCCTCCTCCTATTCTGAGCAGNNNNGNNNATTJACCAGCAGC-3′), which are composed of 454 Lib-L primer B (underlined) and the universal bacterial primer 8F (italic); and the modified primer 534R (5′-CCCTCCTCCATGGGAGTATTTACGAGTCTGAGG-3′), which is a composite of 454 Lib-L primer A (underlined), a universal 6- and 7-base barcode (Ns), and the broad-range bacterial primer 534R (italics). Cycling conditions were 94°C for 3 min, followed by 23 cycles (cycling and fecal samples), 25 cycles (ileum content samples), and 30 cycles (ileum wall samples) of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Replicate PCRs were pooled, and amplicons were purified using the QIAquick PCR Purification kit (QIAGEN). PCR products
were sequenced on a 454 GS FLX Titanium platform according to the 454 Roche recommended procedures. Sequences have been deposited in the Sequence Read Archive of NCBI under submission number SRA052529.

**Sequence analysis.** Sequence data were compiled and processed using mothur (Schloss et al., 2009). Sequences were converted to standard FASTA format. Sequences shorter than 300 bp, containing undetermined bases or homopolymer stretches longer than 8 bp, with no exact match to the forward primer or a barcode, or that did not align with the appropriate 16s rRNA variable region were not included in the analysis. Using the 454 base quality scores, which range from 0–40 (0 being an ambiguous base), sequences were trimmed using a sliding-window technique, such that the minimum mean quality score over a window of 50 bases never dropped below 30. Sequences were trimmed from the 3′ end until this criterion was met. Sequences were aligned to the 16s rRNA gene using as template the SILVA reference alignment (Pruesse et al., 2007) and the Needleman-Wunsch algorithm with the default scoring options. Potentially chimeric sequences were removed using the ChimeraSlayer program (Haus et al., 2011). To minimize the effect of pyrosequencing errors in overestimating microbial diversity (Huse et al., 2010), rare abundance sequences that differ in one or two nucleotides from a high abundant sequence were merged to the high abundant sequence using the pre.cluster option in mothur. Sequences were grouped into OTUs using the mean neighbor algorithm. Sequences with distance-based similarity of 97% or greater were assigned to the same OTU. OTU-based microbial diversity was estimated by calculating the Shannon diversity index or the Chao index (Magurran, 2004). Phylogenetic classification was performed for each sequence using the Bayesian classifier algorithm described by Wang et al. (2007) with the bootstrap cutoff of 60%. In most cases, classification could be assigned to the genus level. Sequences that were classified as Streptophyta, a Viridineae plantae found in mouse feces (Ubeda et al., 2010), were also excluded from the analysis.

When comparing the sequences from this study (Fig. 8) with the sequences from Vijay-Kumar et al. (2010), only the region of the 16s rRNA that was shared between both datasets of sequences (V1-V2) was used for the analysis. To increase the overlapping region within sequences from different experiments, and because our sequences were obtained starting in the V3 region, only those sequences from our study that were longer than 350 bp, instead of 300 bp as described above, were included in this analysis. Most of the sequences were longer than 350 bp.

**Tree building and UniFrac analysis.** A phylogenetic tree was inferred using clearcut (Sheneman et al., 2006) on the 16s rRNA sequence alignment generated by mothur. Unweighted or weighted UniFrac was run using the resulting tree. PCoA was performed on the resulting matrix of distances generated by mothur. Unweighted or weighted UniFrac was run using the 16s rRNA sequence alignment. To increase the overlapping region within sequences from different regions, only those sequences from our study that were longer than 350 bp, instead of 300 bp as described above, were included in this analysis. Most of the sequences were longer than 350 bp.

**Statistical analysis.** To determine whether there were statistically significant differences in bacterial taxa between KO mice and their WT littermates, bacterial taxa with less than five mean count in both conditions were removed, and Student’s t test was applied to log2 transformed scaled count data. The count data were rescaled using DESeq R package (Anders and Huber, 2010). To adjust for multiple hypothesis testing, we used the FDR approach by Benjamini and Hochberg (1995) and used fdr.R package. The final results were filtered for p-value <0.05 and an FDR ≤0.1. The same statistical analysis was applied to determine differences between mice from different litters. The two largest litters in each colony were analyzed for differences in their bacterial taxa (litters B1 and B2 for Myd88, litters B and C for TLR2, litters E and F for TLR4, litters B and D for TLR5, and litters A and D for TLR9).

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