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 (Garner 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). Along similar lines, a recent study demonstrated that mice deficient in NLRP6, a component of the inflammasome, were colonized by a microbiota that exacerbated colitis in NLRP6 KO mice and also in WT mice after microbiota transfer (Elinav et al., 2011). Toll-like receptor (TLR) signaling has also been implicated in the regulation of the intestinal microbiota. The cecal microbiota of TLR5-deficient mice, for example, differed from WT littermate controls in >100 bacterial phylotypes (Vijay-Kumar et al., 2010). In another study, MyD88-deficient mice harbored a cecal microbiota with higher levels of Rikenellaceae and Porphyromonadaceae bacterial families (Wen et al., 2008). Although changes in the microbial composition of the microbiota have been associated with genetic mutations in innate immune signaling pathways, marked differences in microbiota of mice in different colonies housed in different institutions on different continents and exposed to different housing and dietary conditions make it difficult to clearly define the influence of innate immune signaling pathways on the microbiota.

In this study, we focused on the role of MyD88 and TLR signaling on the composition of the intestinal microbiota of mice that have been maintained under specific pathogen–free conditions. We used high-throughput sequencing of 16S rRNA genes amplified from small and large intestinal samples to analyze the microbiota of mice deficient in MyD88 or in specific TLRs (TLR2, TLR4, TLR5, and TLR9) and their WT littermate controls. We find that MyD88 or TLR signaling does not detectably alter the composition of the intestinal microbiota under either steady-state conditions or after antibiotic treatment. Substantial differences in the colonizing microbiota of different MyD88- and TLR–deficient mouse colonies were readily detected, but these disparities correlated with membership in a specific mouse litter and not with impaired innate immune signaling. Our experiments suggest that caution must be exercised in the interpretation of microbiota analyses of mutant and WT mice and that meticulous attention to the parental lineage and husbandry of mice is essential.

RESULTS
Impact of MyD88/TLR signaling on the intestinal microbiota
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
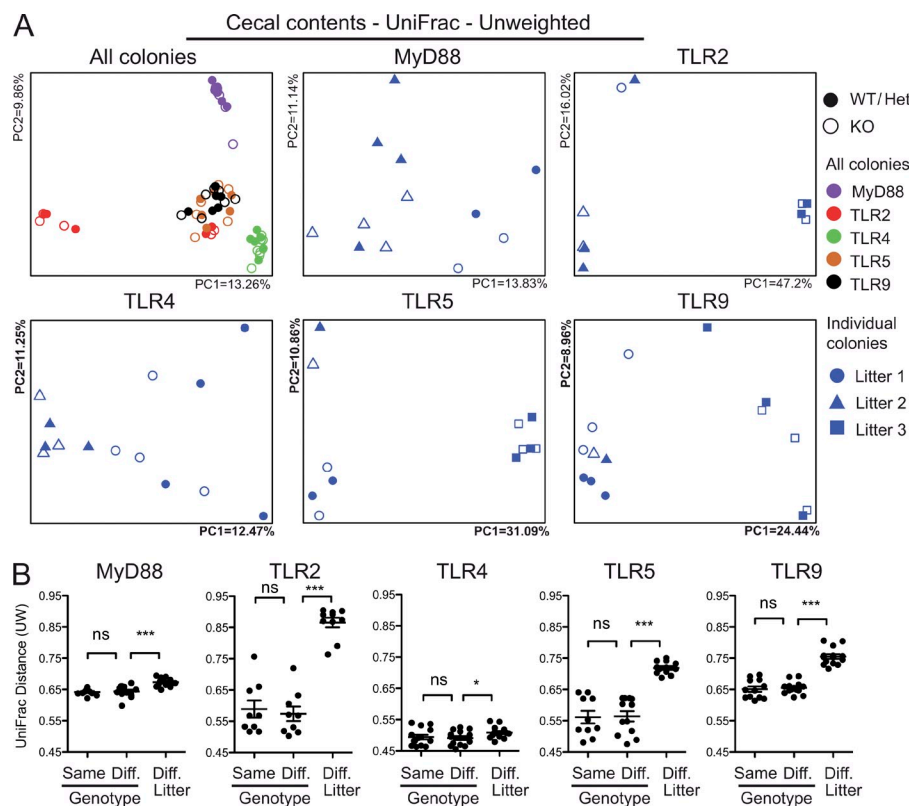
Figure 1. Impact of TLR signaling versus lineage origin on the overall microbial composition of the cecal microbiota. (A) PCoA plot of the unweighted UniFrac analysis of cecal samples of MyD88, TLR2, TLR4, TLR5, or TLR9 KO mouse colonies and their respective WT/Het littermates. Each mouse colony contains five to seven KO and five to seven WT/Het mice and is labeled with a different color, as indicated. When mouse colonies are analyzed separately, the litter origin of each mouse is indicated with a different symbol shape. (B) The mean UniFrac distances for each mouse in a colony versus (a) all 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 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.

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 clustered together, PC1 and PC2 separated mice by their parental origin. Similar results were obtained for TLR4-, TLR5-, and TLR9-deficient mouse colonies upon analysis of different litters (Fig. 1 A). Similar separation of different litters was observed using weighted UniFrac or when samples isolated from the ileal epithelium or ileal lumen were analyzed (not depicted). These results indicate that maternal origin has a greater impact than TLR signaling on the composition of the intestinal microbiota. To demonstrate the relative contributions of genotype versus lineage on the microbiota, we calculated and plotted the mean unweighted

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.

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
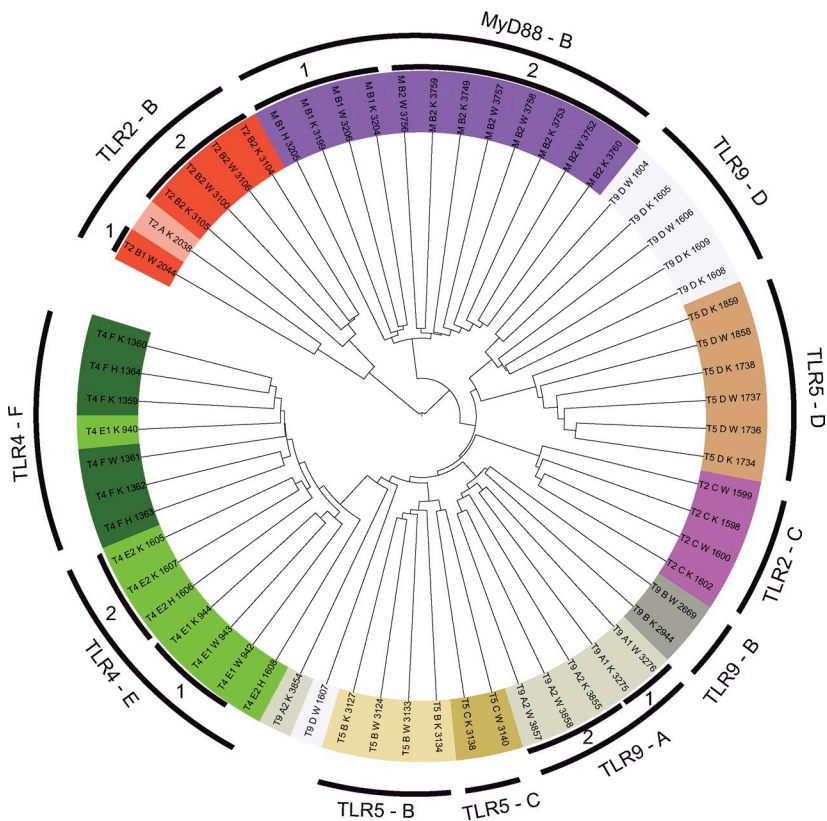

Figure 2. Maternal effect on the microbiota composition. Cecal microbiota samples were clustered by similarity using the UPGMA clustering algorithm on the unweighted UniFrac distance matrix. Colors indicate the mouse maternal origin. Samples were named using the following four-label code: (1) mouse colony (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.

(Fig. 4), and on average, 26.6 OTUs per colony and site were found to be significantly different between litters. To identify microbiota differences resulting from TLR signaling and to avoid differences resulting from maternal origin, we compared KO mice with WT littermates of the same litter. Only litters that contained at least three KO and three WT littermates were included in this analysis, enabling one litter from MyD88 and TLR5 and two litters for TLR4 and TLR9 colonies to be included. Using this approach, we only identified statistically significant differences in one litter from the TLR9 colony, in which the *Olsenella* genus abundance was higher in the ileal lumen and epithelium of KO mice, and *Parasutterella*, *Turicibacter*, and unclassified *Erysipelotrichaceae* abundance were higher in the ileal epithelium of KO mice. At the OTU level, sporadic changes were observed: reduction of OTU257 (Ruminococcaceae) in the cecum of one litter of TLR5 KO mice, reduction of OTU8 (*Lactobacillus*) in the ileal wall of one litter of TLR4 KO mice, and increase of OTU26 (*Olsenella*) in the ileal contents of one litter of 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).

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. Fig. 5 shows the relative amount of the most abundant taxa identified in fecal samples from MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$,
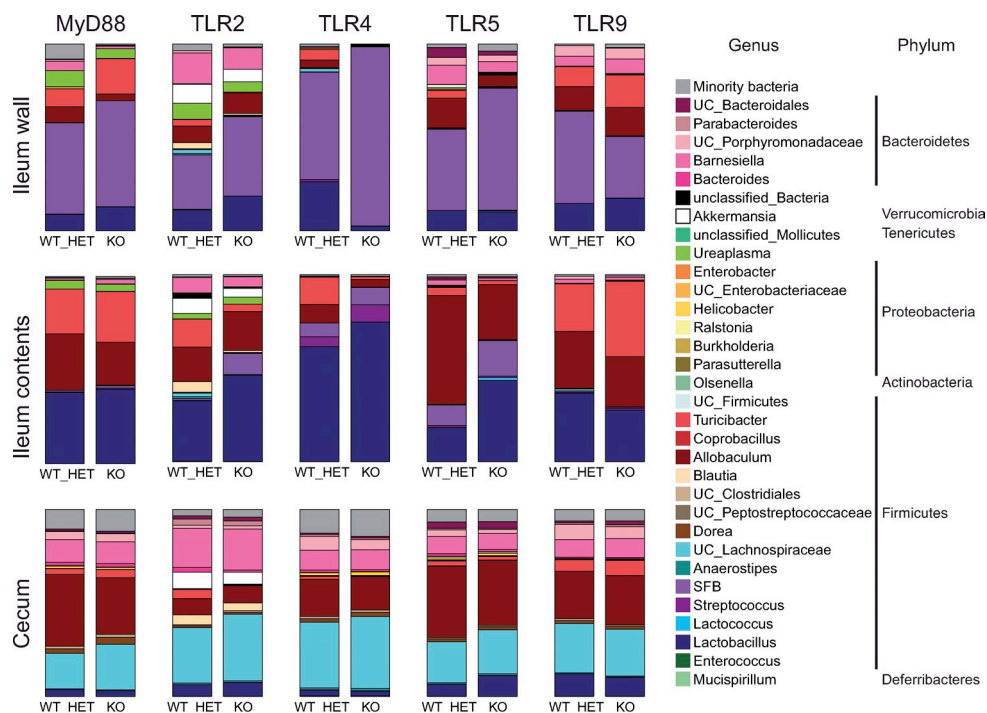

Figure 3. Impact of TLR signaling on the bacterial composition of the intestinal microbiota. Phylogenetic classification of 16S rDNA frequencies in the ileum or cecum samples from colonies of MyD88, TLR2, TLR4, TLR5, or TLR9 KO mice and their respective WT/Het controls. Each bar represents the mean of the microbiota composition from five to seven mice. The most predominant bacterial taxa are shown and labeled with different colors as indicated. Bacterial taxa were obtained by classification of 16s rDNA sequences to the genus level using mothur. In case a sequence could not be classified to the genus level, the closest level of classification to the genus level was given, preceded by UC (unclassified).
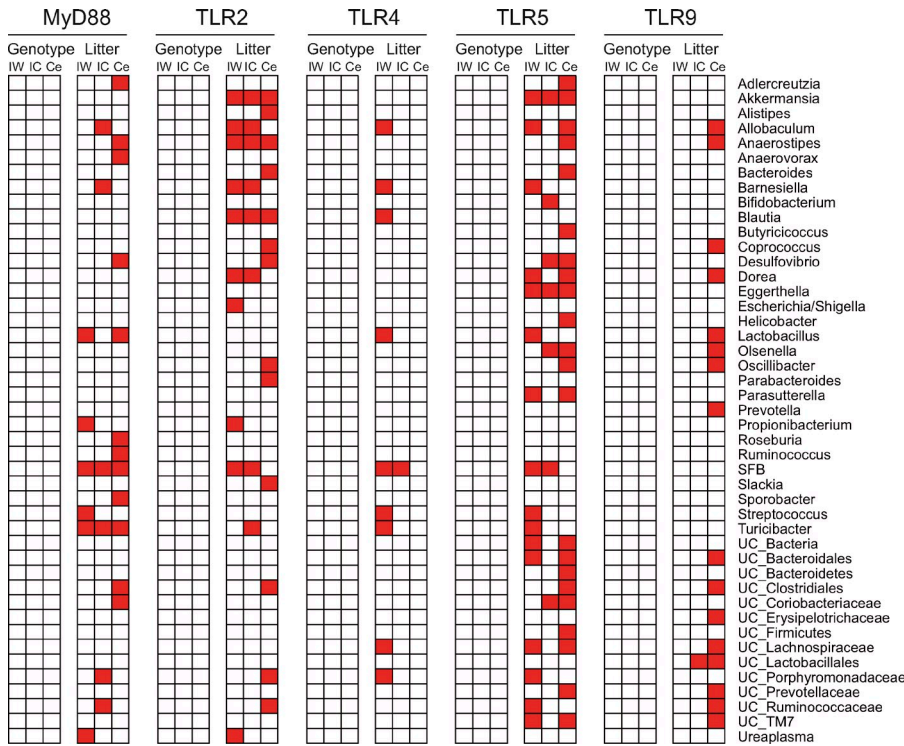
Figure 4. Impact of TLR signaling versus lineage origin on specific bacterial taxa of the intestinal microbiota. Heat map showing statistically significant changes (Student's t test, $P < 0.05$; FDR ≤ 0.1) on different intestinal bacterial taxa (red) between WT/Het and KO mice ($n = 5$–7 mice/genotype) or between different litters within a mouse colony ($n = 4$–8 mice/litter). Bacterial taxa were obtained by classification of 16s rDNA sequences to the genus level using mothur. In case a sequence could not be classified to the genus level, the closest level of classification to the genus level was given, preceded by UC (unclassified). Only those taxa that were found to be statistically significant at least once are shown. Ce, cecal contents; IC, ileum contents; IW, ileum wall.

although it never returned to pretreatment levels. We used the Student's t test ($P < 0.05$) to determine whether the microbial diversity in KO and WT littermates differed significantly. This analysis detected a slightly higher diversity in MyD88$^{-/-}$ mice 14 d after recovery. Calculation of microbial richness using the Chao index did not detect significant differences after vancomycin treatment of KO and WT littermates (Fig. 6 B).

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 *Helicobacter* was observed, whereas expansion of *Parabacteroides* 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 recovering from antibiotic treatment differed significantly after applying FDR criteria (≤0.1).

We and others have shown that antibiotic treatment diminishes intestinal microbial diversity (Dethlefsen et al., 2008; Ubeda et al., 2010). It is unclear, however, whether TLR signaling contributes to the decrease in microbial diversity after antibiotic treatment. To address this question, the Shannon diversity index was calculated for fecal samples (Fig. 6 A). As expected, microbial diversity diminished 7 d after vancomycin treatment and slightly increased after antibiotic withdrawal,

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
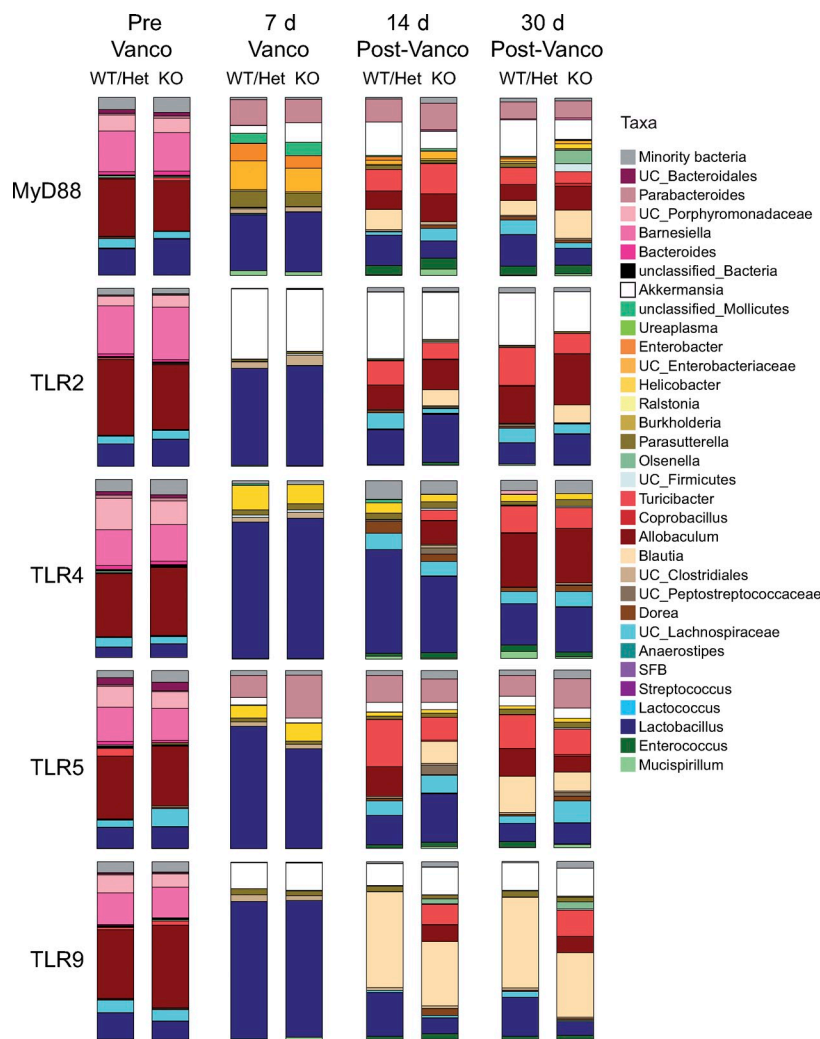
Figure 5. Impact of TLR signaling on the fecal microbiota composition after antibiotic treatment.
MyD88, TLR2, TLR4, TLR5, or TLR9 KO mice and WT/Het littermate controls were treated with vancomycin (Vanco) for 7 d on their drinking water. Fecal samples were obtained before treatment, 1 wk after vancomycin 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.

demonstrates that deficiency in MyD88, TLR2, TLR4, TLR5, or TLR9 does not result in significant changes in the composition, richness, or diversity of the microbiota associated with the ileal epithelium, ileal lumen, or cecal lumen. Our experiments did not detect changes in the microbiota that could be attributed to TLR deficiency under homeostatic conditions or after recovery from high-dose, luminal antibiotic treatment with vancomycin. In contrast to TLR signaling, maternal origin seems to be the major factor driving the microbiota differences observed in both MyD88- and individual TLR–deficient mouse colonies. Our results agree with a previous study by Turnbaugh et al. (2009a), which showed that individuals from the same family have a more similar microbiota than unrelated individuals. Our results extend the results of Wen et al. (2008) by demonstrating that maternal origin had a greater effect on the microbiota composition than Myd88 deficiency. Interestingly, some differences in the microbiota could be observed in mice with the same maternal origin but from different litters (MyD88 colony). Although the microbiota of adult mice remains stable, it is possible that slight temporal variations in the maternal microbiota could lead to differences in the offspring microbiota.

Vancomycin–resistant *Enterococcus* (VRE) is an important pathogen that can cause systemic infections in immunocompromised patients. We have shown that intestinal domination of VRE can lead to bacterial sepsis in allogeneic transplant patients (Ubeda et al., 2010). VRE colonization of the small intestine is facilitated by antibiotic-mediated depletion of the commensal flora, and stimulation of TLR4 by oral LPS or TLR5 by systemic flagellin enhances resistance to VRE colonization (Brandl et al., 2008; Kinnebrew et al., 2010). These results indicate that TLR-mediated signals can impact bacterial colonization of the gut lumen, particularly by exogenously administered organisms that are not normal components of the microbiota. Although our experiments demonstrate that the prevalence of commensal microbial populations is

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 microbial communities on 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 TLR–deficient littermate control mice, in which offspring resulted from crosses of mice heterozygous for each deletion, however,
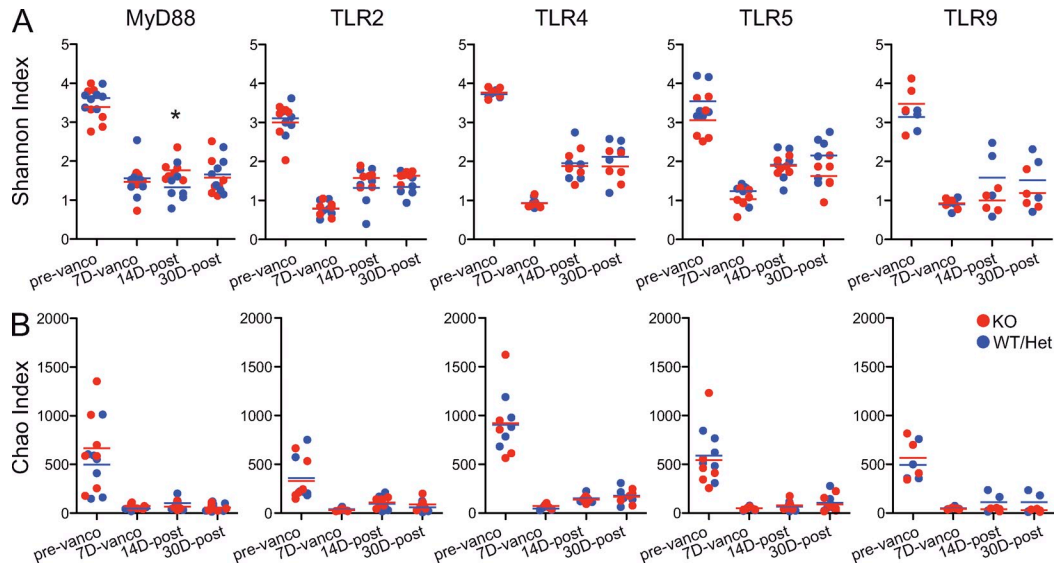
Figure 6.   Impact of TLR signaling on the diversity and richness of the intestinal microbiota after antibiotic treatment. (A and B) Myd88, TLR2, TLR4, TLR5, or TLR9 KO mice and WT/Het littermate controls were treated with vancomycin (vanco) for 7 d on their drinking water. Fecal samples were obtained 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).

not detectably affected by TLR signaling under homeostatic conditions or after antibiotic treatment, it is likely that TLR–mediated effector mechanisms contribute to the inhibition of intestinal colonization by exogenous invaders, thereby contributing to colonization resistance.

MyD88 signaling has been implicated in the development of diabetes in the nonobese diabetic (NOD) mouse model (Wen et al., 2008). Interestingly, germ-free, MyD88-deficient NOD mice are more susceptible to developing diabetes than bacterially colonized MyD88-deficient NOD mice. Characterization of the microbiota of MyD88-deficient and WT littermate control mice revealed a significant decrease in the ratio of bacteria belonging to the Firmicutes and Bacteroidetes phyla in MyD88-deficient mice and increased colonization of MyD88-deficient mice with bacteria belonging to the Rikenellaceae and Porphyromonadaceae families. In another study, an increase in the relative abundance of SFB was observed in MyD88$^{-/-}$ mice, primarily in the jejunum and ileum, although no littermate controls were used (Larsson et al., 2012). We did not detect similar differences in our colonies, although those bacterial taxa were present in the microbiota of mice used in our study. It is possible that different bacterial species within the families Rikenellaceae and Porphyromonadaceae inhabit the intestines of mice from Wen et al. (2008) and our studies and might explain the differences in our results. Our finding that the ileal and cecal contents were similar in WT and MyD88-deficient littermate mice agrees with a recent study comparing the microbiota of the ileal lumen of MyD88-deficient and WT mice (Vaishnava et al., 2011). Vaishnava et al. (2011) showed

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.

TLR5 signaling is implicated in the pathogenesis of metabolic syndrome, which includes obesity, insulin-resistant diabetes, hyperlipidemia, and hepatic steatosis (Vijay-Kumar et al., 2010). The metabolic syndrome persisted in TLR5-deficient mice that had been rederived in mice obtained from the Jackson
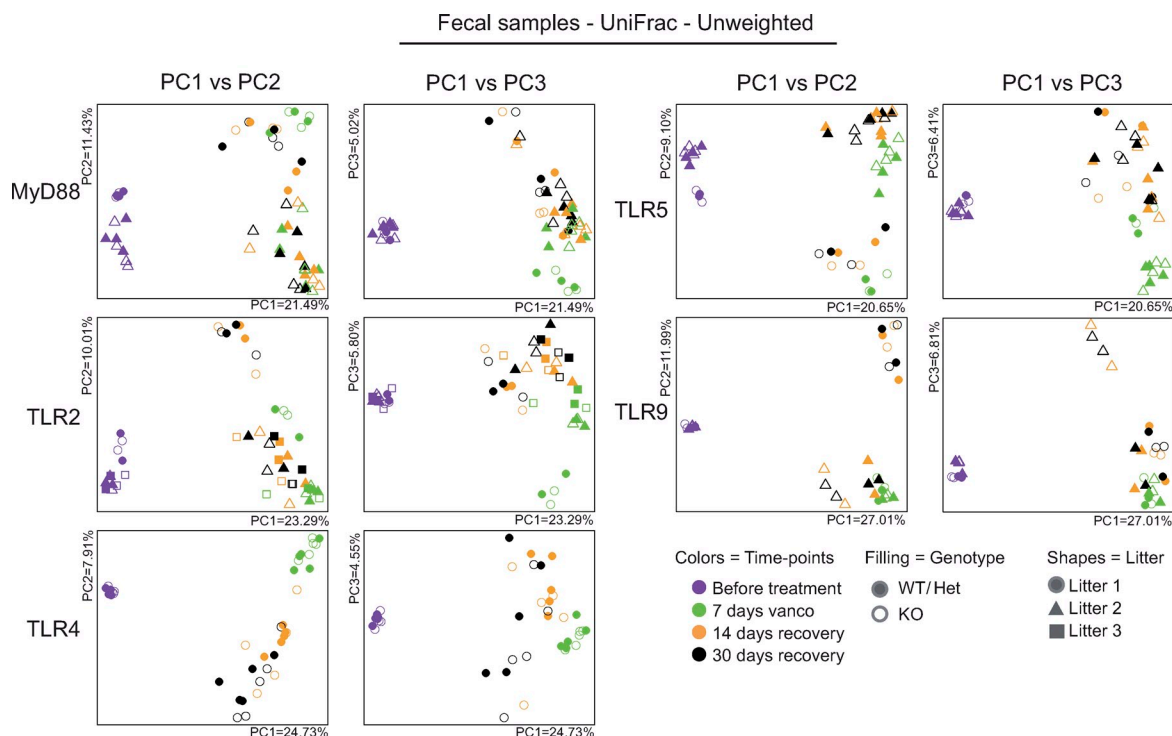
Fecal samples - UniFrac - Unweighted

Figure 7. Impact of TLR signaling versus lineage origin on the overall microbial composition of the fecal microbiota after antibiotic treatment. PCoA plot from the unweighted UniFrac analysis of fecal samples from colonies of MyD88, TLR2, TLR4, TLR5, or TLR9 KO mice and their respective WT/Het littermates. Mice were treated with vancomycin (vanco), and fecal samples were obtained before treatment, 1 wk after vancomycin treatment, or 2 and 4 wk after antibiotic withdrawal. Symbol shapes denote the different litters of mice within a colony. Each mouse colony contains four to seven KO mice and four to seven WT/Het mice.

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
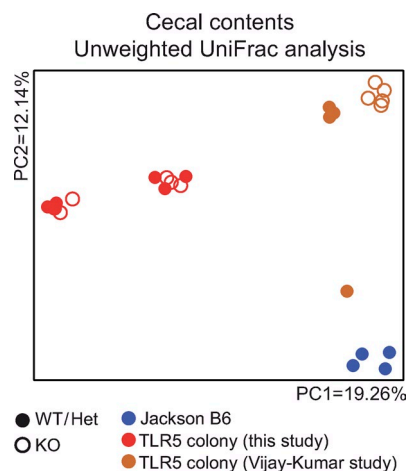

Figure 8. Husbandry impact on the effect of TLR5 signaling on the microbiota composition. PCoA plot from the unweighted UniFrac analysis of cecal samples from WT B6 mice from the Jackson Laboratory maintained for 2 wk in our animal facility (*n* = 4), TLR5-deficient mice raised in our animal facility (*n* = 6) and their respective WT littermate controls (*n* = 6), and TLR5-deficient mice from Vijay-Kumar et al. (2010; *n* = 5) and their respective WT littermate controls (*n* = 4).

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 (altered 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 microbiota analysis because our previous results have shown that the mouse intestinal microbiota becomes stable beyond at least 6 wk of age (Buffie et al., 2012). Two sequential litters obtained from the same MyD88$^{+/-}$ breeding pair were analyzed. A similar strategy was used to compare the microbiota of TLR2$^{-/-}$, TLR4$^{-/-}$, TLR5$^{-/-}$, and TLR9$^{-/-}$ with their respective WT (either +/+ or +/−) littermate controls. In this case, litters were obtained from parallel breeding pairs. Parent origin is indicated with a different letter in the figures and text.

MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice that were used to generate the aforementioned colonies were obtained in 2000 from the Medzhitov laboratory at Yale University. TLR5$^{-/-}$ and TLR9$^{-/-}$ mice were obtained from the Flavell laboratory at Yale University in 2004 and from the Institut für Mikrobiologie

Klinikum recht der Isar in 2003, respectively. The different KO mice were initially backcrossed to Jackson Laboratory C57BL/6 mice shortly after arrival at our animal facility and have been bred and maintained in separate cages. All mouse colonies have been maintained in the same room and generally in the same rack. They were maintained by the same personnel that changed their gloves and used sterile utensils to prevent transfer of microbes between cages. All mice were fed irradiated food and maintained in autoclaved cages. Both males and females were analyzed to increase the number of littermates. Although gender may influence microbial composition, analysis of littermate mice suggested that differences by gender are likely negligible.

For experiments involving antibiotic treatment, mice were individually housed in the same conditions described above. 5–7 wk after weaning, mice were treated for 1 wk with drinking water containing vancomycin (1 g/liter).

Sample collection and DNA extraction. To avoid the confounding effects of co-housing on the diversity of the intestinal microbiota, for each 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).

Total genomic DNA was extracted from the ileum wall samples using a combination of 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 MgCl$_2$, 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 (Buffie et al., 2012): a modified primer 8F (5′-<u>CCTATCCCCTGTGT-GCCTTGGCAGTCTC</u>*AGAGAGTTTGATCCTGGCTCAG*-3′), composed of 454 Lib-L primer B (underlined) and the universal bacterial primer 8F (italics); and the modified primer 534R (5′-<u>CCATCTCATC-CCTGCGTGTCTCCGACTCAG</u>NNNNNNNN*ATTACCGCGGCT-GCTGG*-3′), which is a composite of 454 Lib-L primer A (underlined), a unique 6- or 7-base barcode (Ns), and the broad-range bacterial primer 534R (italics). Cycling conditions were 94°C for 3 min, followed by 23 cycles (cecum 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 rage 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 (Haas 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 Viridiplantae found in mouse feed (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 between each pair of samples.

To compare the different contribution of TLR defects or family origin to the microbiota composition (Fig. 1 B), the mean UniFrac distances for each mouse in a colony versus (a) all 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 were calculated and plotted.

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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