

ORIGINAL ARTICLE

Host adaptive immunity alters gut microbiota

Husen Zhang¹, Joshua B Sparks², Saikumar V Karyala³, Robert Settlage³ and Xin M Luo⁴
¹Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA, USA; ²Carilion School of Medicine, Roanoke, VA, USA; ³Virginia Bioinformatics Institute, Blacksburg, VA, USA and ⁴Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, VA, USA

It has long been recognized that the mammalian gut microbiota has a role in the development and activation of the host immune system. Much less is known on how host immunity regulates the gut microbiota. Here we investigated the role of adaptive immunity on the mouse distal gut microbial composition by sequencing 16S rRNA genes from microbiota of immunodeficient Rag1^{-/-} mice, versus wild-type mice, under the same housing environment. To detect possible interactions among immunological status, age and variability from anatomical sites, we analyzed samples from the cecum, colon, colonic mucus and feces before and after weaning. High-throughput sequencing showed that Firmicutes, Bacteroidetes and Verrucomicrobia dominated mouse gut bacterial communities. Rag1^{-/-} mice had a distinct microbiota that was phylogenetically different from wild-type mice. In particular, the bacterium *Akkermansia muciniphila* was highly enriched in Rag1^{-/-} mice compared with the wild type. This enrichment was suppressed when Rag1^{-/-} mice received bone marrows from wild-type mice. The microbial community diversity increased with age, albeit the magnitude depended on Rag1 status. In addition, Rag1^{-/-} mice had a higher gain in microbiota richness and evenness with increase in age compared with wild-type mice, possibly due to the lack of pressure from the adaptive immune system. Our results suggest that adaptive immunity has a pervasive role in regulating gut microbiota's composition and diversity.

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Introduction

The mammalian gut is one of the most densely colonized habitats with trillions of microorganisms known as the microbiota (Ley *et al.*, 2008). The microbiota have coevolved with the host and have a key role in host's metabolism and immunity (Hooper *et al.*, 2001; Backhed *et al.*, 2004; Gill *et al.*, 2006; Round and Mazmanian, 2009; Klaenhammer *et al.*, 2012). Imbalance in the gut microbiota composition has been associated with diseases (Ley *et al.*, 2005; Turnbaugh *et al.*, 2008; Vijay-Kumar *et al.*, 2010; Blaser, 2010). External factors, such as diet and antibiotics, have been shown to have an important role in shaping gut microbiota. For example, transition from milk to solid food in infants correlated an increase in Bacteroidetes (Koenig *et al.*, 2010). In addition, gnotobiotic mice transplanted with human gut microbiota can be modulated with the diet (Turnbaugh *et al.*, 2009). Antibiotic use, on the other

hand, has been shown as a key factor to the perturbation of gut microbiota (Young and Schmidt, 2004; Dethlefsen *et al.*, 2008).

Compared with modulation of gut microbiota by external factors, information is limited on how host immunity regulates its gut microbiota (Willing *et al.*, 2011; Brown *et al.*, 2013). Recent findings suggest an important role for the innate immune system. In particular, toll-like receptor (TLR5), which recognizes the bacterial flagellin to activate innate immune response, has been shown to alter colonic microbiota at the species level (Vijay-Kumar *et al.*, 2010). In contrast, TLR2 and TLR4, which mainly recognize peptidoglycan and lipopolysaccharide, respectively, have no apparent effect on gut microbiota (Loh *et al.*, 2008). In addition, deficiency of MyD88, a signaling adapter required for all toll-like receptors except TLR3, has been reported to alter gut bacterial diversity with an expanded population of segmented filamentous bacteria in the small intestine (Larsson *et al.*, 2011). In non-obese diabetic mice, MyD88 deficiency was further shown to change gut microbiota that conferred protection against developing Type 1 diabetes (Wen *et al.*, 2008). Another example of microbiota-regulating component of innate immunity is Interleukin-22-producing innate lymphoid cells, which were shown to selectively control colonization of

Correspondence: XM Luo, Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Tech, Building 142, 295 Duck Pond Drive, Blacksburg, VA 24061, USA or H Zhang, Department of Civil and Environmental Engineering, Virginia Tech, 1145 Perry Street, 414 Durham, Blacksburg, VA 24061, USA.

E-mail: xinluo@vt.edu or husen@vt.edu

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Alcaligenes species to prevent systemic inflammation (Sonnenberg *et al.*, 2012).

It has been hypothesized that the adaptive immune system evolved in vertebrate animals in order to memorize complex assemblages of beneficial microbiota (McFall-Ngai, 2007). It is thus conceivable that deficiencies in the host adaptive immune system would result in an altered microbiota. Indeed, such deficiency can lead to a markedly elevated level of bacterial flagellin (Cullender *et al.*, 2013), which is normally kept low presumably by host immunity. In addition, mice deficient in recombination-activating gene 2 (Rag2) or activation-induced cytidine deaminase had increased segmented filamentous bacteria in the small intestine (Suzuki *et al.*, 2004). Both mutations lead to the lack of IgA (immunoglobulin A) in the intestine, which provides 'an immunological buffer' between the host and microbiota (Brown *et al.*, 2013). Besides IgA (Cebra, 1999; Suzuki *et al.*, 2004; Macpherson and Uhr, 2004; Suzuki and Fagarasan, 2008), T-cell-mediated responses are also hypothesized to shape microbiota in the intestine but evidence is scarce. Importantly, the role of mature lymphocytes on the composition and diversity of gut microbiota is not known.

Microbiota vary with host age (Palmer *et al.*, 2007; Koenig *et al.*, 2010), diet (Cani *et al.*, 2007) and luminal versus mucosal localization (Zoetendal *et al.*, 2002; Eckburg *et al.*, 2005). Environmental gradients created by water absorption and pH variability along the distal intestine could also affect microbiota composition (DiBaise *et al.*, 2008). Thus, in this study, we aim to investigate the role of adaptive immunity on mouse distal gut microbiota with a carefully controlled study design that allows us to discern any interactions between age, intestinal location and immunological status. For this purpose, we employed the classic, non-leaky Rag1^{-/-} mouse model that lacks all mature lymphocytes. Both wild-type (Rag1^{+/+}) and mutant (Rag1^{-/-}) mice were given the same sterile diet and water, and housed in the same clean environment. We took samples before and after weaning in order to detect possible interactions between immunological status and age. For each immunological status and age, we analyzed microbiota compositions from multiple anatomical sites including cecum, colon, colonic mucus and feces. Finally, to explicitly investigate the role of mature lymphocytes on microbiota, we performed adoptive transfer to Rag1^{-/-} mice using Rag1^{+/+} bone marrow that replenished the adaptive immune system.

Materials and methods

Experimental design

Both Rag1^{+/+} and Rag1^{-/-} mice were of C57BL/6 background. After being purchased from The Jackson Laboratory (Bar Harbor, ME, USA), both

strains were housed in the same room and on the same shelf of a maximum barrier, specific pathogen-free facility at College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA. To ensure adaptation to identical housing environment, both strains were bred in-house for at least three generations with sterilized individually ventilated caging, and received sterilized drinking water and feed (2918-Irradiated Teklad Global 18% Protein/6% Fat Rodent Diet, Harlan Laboratories, Indianapolis, IN, USA) prior to the initiation of the study. The same housing condition was used in the entire study. For adoptive transfer, the bone marrow of 6-week-old female Rag1^{+/+} or Rag1^{-/-} mice was injected intravenously into age-matched female Rag1^{-/-} mice of the same litter. Using recipient mice of the same litter was intended to reduce the confounding effect of maternal microbiota transmission on pups' gut flora (Ubeda *et al.*, 2012). The experiment was repeated once to achieve a sample size of six in each group. Both litters of recipient mice were from the same Rag1^{-/-} dam. Briefly, total bone marrow cells were collected from femurs and tibias of Rag1^{+/+} or Rag1^{-/-} mice, removed of red blood cells, washed and resuspended in sterile phosphate-buffered saline. Recipient Rag1^{-/-} mice from the same litter were anesthetized with isoflurane carried in oxygen and retro-orbitally injected with Rag1^{+/+} or Rag1^{-/-} bone marrow cells (2 × 10⁶ cells in 150 µl phosphate-buffered saline per mouse). The recipient mice were housed for 8 weeks until sample collection at 14 weeks of age. To retain gut microbiota, the standard procedure of antibiotics treatment in bone marrow transplantation was omitted.

Microbiota sampling, DNA extraction and PCR

Microbiota samples were collected from cecum, colon and colonic mucus within 20 mins after euthanasia. Cecal content was collected by manual extrusion using a sterile pipette tip. The colon was carefully opened longitudinally with a pair of sterile scissors. Colonic content was gently picked up with tweezers. Colonic mucus was then collected by gently scraping off the colonic wall. To avoid cross-contamination, each microbiota sample was collected using a new pair of sterile tweezers. For T2 only, feces were picked up directly from individual cages. (For T1, pups were housed in the same cage with their mothers, making the collection of feces from individual pups impossible.) For adoptive transfer, microbiota samples were collected from the lumen of the colon. All samples were stored at -80 °C till being processed at the same time. DNA was extracted with the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). The V4 region (252 bp) of 16S rRNA gene was PCR-amplified with 515F and 12-base GoLay barcoded 806R primers (Caporaso *et al.*, 2012). The purified amplicons were sequenced with a MiSeq sequencer (Illumina, San Diego, CA, USA).

Taxonomy assignments and community structure analyses
Sequencing reads were processed with Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010). High-quality reads with Phred score ≥ 20 (corresponding to a sequencing error rate ≤ 0.01) were first checked for chimeras with USEARCH61 (Edgar, 2010). Chimeric sequences were removed from further analysis. Nonchimeric sequences were mapped into operational taxonomic units (OTUs) against the Greengene reference sequences (version 2013.5) with the program UCLUST (Edgar, 2010). Bacterial taxonomy was assigned by using a naive Bayes classifier (Wang *et al.*, 2007) trained with the Greengenes taxonomy with over 200 000 reference sequences (McDonald *et al.*, 2012). Abundant taxa were classified to the species level by using BLAST (Altschul *et al.*, 1997) against the Greengenes data set. A phylogenetic tree was constructed (Price *et al.*, 2010) from PyNAST-aligned sequences representing each OTU. For beta diversity calculations, all microbiota were randomly sampled with 13 000 sequences to achieve even sampling. Principle coordinates were calculated from unweighted UniFrac metrics (Lozupone and Knight, 2005). Alpha diversity metrics, including observed species defined as OTUs with 97% or higher similarity, phylogenetic diversity (Faith, 1992) and Shannon index, and equitability were calculated using QIIME.

Nucleotide accession numbers

Sequences determined in this study were deposited in the Metagenomics RAST server (Meyer *et al.*, 2008) under the accession number 4547766.3.

Total bacteria measurement by real-time PCR

Total bacteria were measured by using real-time PCR with domain-specific primers F340 and R514 (Wlodarska *et al.*, 2011). Five-point standard curves were generated by using *Lactobacillus rhamnosus* (ATCC 7469) genomic DNA. Real-time PCR was performed using iTaq Universal Supermixes (Bio-Rad, Hercules, CA, USA) on an Applied Biosystems (Foster City, CA, USA) 7500 cycler with the program: one cycle at 95 °C for 5 mins, followed by 40 cycles of 94 °C for 15 s and 63 °C for 45 s.

Flow cytometry analysis

Mononuclear cells were isolated from the spleen and colonic lamina propria as described previously (Hur *et al.*, 2012). Cells after staining with fluorochrome-labeled antibodies (eBioscience, San Diego, CA, USA) were analyzed with the FACS Aria (BD Biosciences, San Jose, CA, USA).

Statistics

The significance of microbiota grouping by Rag1 status and by age was determined by permutational

multivariate analysis methods PERMANOVA and PERMDISP (Anderson, 2001). Unpaired Student's *t*-test was performed in Microsoft Excel 2011. Mann-Whitney *U*-test, one-way and two-way analysis of variance were performed by using Prism (GraphPad, La Jolla, CA, USA) and R version 3.0.2.

Results

Rag1 status as a source of variation in gut microbiota community structure

We compared Rag1^{+/+} and Rag1^{-/-} mice of the same genetic background to determine the effect of Rag1 and adaptive immune system on gut microbiota. Mice lacking the adaptive immune system are often housed at commercial vendors in maximum barrier facilities. Wild-type mice, on the other hand, are usually raised in standard barrier rooms. This may result in distinct baseline microbiota for these strains, known as the cage effect. Several experimental approaches could be used to eliminate the cage effect as a potential confounding factor in our study. The first option was to breed heterozygous (Rag1^{+/-}) animals and compare Rag1^{+/+} and Rag1^{-/-} littermates. The second option was to co-house Rag1^{+/+} and Rag1^{-/-} mice to allow coprophagy, which could be used to equilibrate gut microbial diversity in co-housed animals (Antonopoulos *et al.*, 2009). The third option was to adapt Rag1^{+/+} animals to the same housing conditions as Rag1^{-/-} mice (maximum barrier and sterilized caging, water and feed). Because we planned to involve preweaning pups and their gut microbiota might be affected by immunoglobulins in milk and placenta, the third option was chosen as it restricted Rag1^{-/-} pups to Rag1^{-/-} dams (that is, no immunoglobulins from milk or placenta). Therefore, herein we will describe the changes of gut microbiota in the absence of the entire adaptive immunity that includes maternal immunoglobulins.

Microbiota samples from cecal, colonic and mucosal contents at two different ages, 14 days post birth (T1) and 28 days postweaning (T2), were analyzed. For T2 when mice were individually caged, fecal samples were also collected and analyzed. High-throughput sequencing of the 42 samples yielded 9 342 844 reads. After quality trimming and chimera removal, the final data set contained 7 330 205 nonchimeric high-quality sequences and was used for taxonomy assignments and diversity analyses.

Differences between microbiota samples were calculated by using the UniFrac metrics, which measures phylogenetic dissimilarities between microbial communities (Lozupone and Knight, 2005). Principal coordinate analysis based on UniFrac metrics showed a separation of Rag1^{+/+} and Rag1^{-/-} samples along the first two axes that explained 17% and 13% of data variation, respectively (Figure 1a). Rag1 status as a significant source of variation was confirmed by a nonparametric

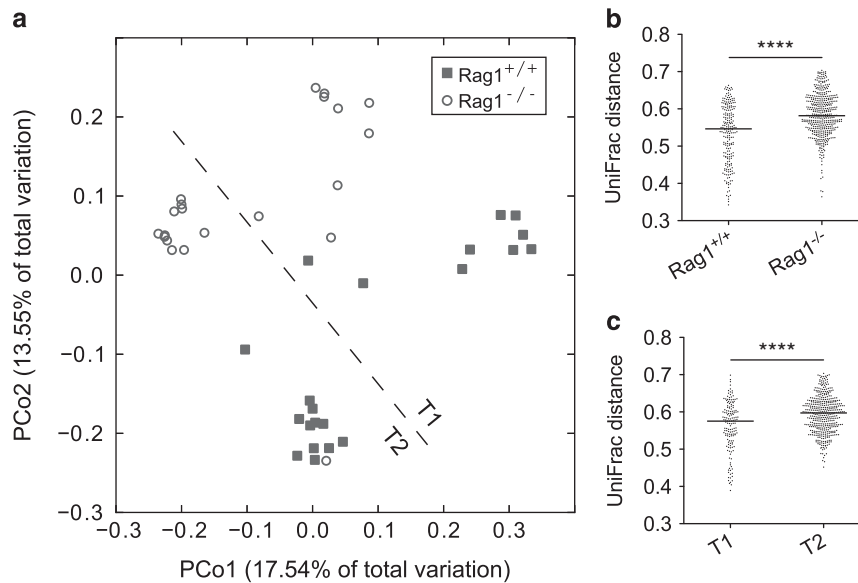


Figure 1 Microbiota community structures explained by *Rag1* mutation and animal age. (a) Principal coordinate analysis based on unweighted UniFrac metrics. *Rag1*^{+/+} and *Rag1*^{-/-} samples are coded as black squares and open circles, respectively. The dashed line separates samples based on the age of the animals (T1: 14 days post birth; T2: 28 days postweaning). (b) Scatterplots of distances between *Rag1*^{+/+} microbiota themselves ($n=210$) and between *Rag1*^{+/+} and *Rag1*^{-/-} microbiota ($n=210$). The median is plotted as a horizontal line. (c) Distances between T1 microbiota themselves ($n=153$) and between T1 and T2 microbiota ($n=432$), with the horizontal line as the median. Statistical comparison was based on nonparametric Mann–Whitney *U*-test (**** $P<0.0001$).

permutational multivariate analysis, PERMANOVA ($P<0.001$). We further confirmed that *Rag1*^{+/+} and *Rag1*^{-/-} samples differed because of their coordinates and not their relative dispersions by the PERMDISP test ($P=0.438$) that showed the two groups had similar dispersion. In contrast, although samples from two different ages differ from each other based on the PERMANOVA test ($P<0.001$), dispersion was a significant factor that caused this difference (PERMDISP, $P<0.001$). Samples from different anatomical locations of the intestine were not significantly different ($P=0.099$). Importantly, the average UniFrac distance between *Rag1*^{+/+} and *Rag1*^{-/-} samples was larger than that within *Rag1*^{+/+} samples (Figures 1b, $P<0.0001$), indicating significantly greater phylogenetic difference between *Rag1*^{+/+} and *Rag1*^{-/-} samples than within *Rag1*^{+/+} themselves. Likewise, larger average UniFrac distance between T1 and T2 samples than within T1 samples supported change of communities with sampling age (Figures 1c, $P<0.0001$).

Bacterial taxonomy comparisons

Bacterial taxa showed variations with treatment, age and anatomical site (Figure 2a). Firmicutes, Bacteroidetes and Verrucomicrobia, collectively representing over 90% of total sequences in each subject, dominated the gut microbiota. Actinobacteria, Proteobacteria and Tenericutes were present as minor constituents. The major components at the taxonomic Order level were Clostridiales, Lactobacillales, Bacteroidales and Verrucomicrobiales (Figure 2a). It is notable that total bacterial numbers

did not vary significantly with immunodeficiency (Supplementary Figure S1). Regardless of *Rag1* status, 1 g of cecal or colonic content contained 10^{10} – 10^{11} copies of 16 S rDNA. One gram of mucus had $10^{7.5}$ – $10^{9.5}$ copies of 16 S rDNA. One gram of feces contained $10^{8.6}$ – 10^{10} copies of 16 S rDNA.

Several taxa were markedly affected by *Rag1* deficiency. *Rag1*^{-/-} mice had significantly lower colonic Lactobacillales and Enterobacteriales than the controls had at T1 (Figure 2b). The phenomenon was absent at T2 (Supplementary Table S1), suggesting that the association was age-dependent. At T2, *Rag1*^{-/-} mice had significantly more Verrucomicrobiales ($P<0.05$) in colonic content and feces compared with *Rag1*^{+/+} mice (Figure 2b). The same trend was observed at T1 for cecal content, colonic content and colonic mucus (Figure 2a, shown in pink color), but the difference was not statically significant (Supplementary Table S1) due to variations within the *Rag1*^{-/-} group.

The age of the mice also affected microbiota taxa. Lactobacillales decreased from T1 to T2 in the colon of *Rag1*^{+/+} mice (Figure 2c), consistent with previous reports (Pantoja-Feliciano *et al.*, 2013) and due to the diet change from milk to solid food before and after weaning. Because *Rag1*^{-/-} colons did not have much Lactobacillales to begin with (Figure 2b), the decrease from T1 to T2 was less obvious (Figure 2a and Supplementary Table S2). Analysis of other major components such as Clostridiales and Bacteroidales also revealed changes over time (Supplementary Table S2), reflecting major changes in microbiota before and after weaning. In particular, a shift from

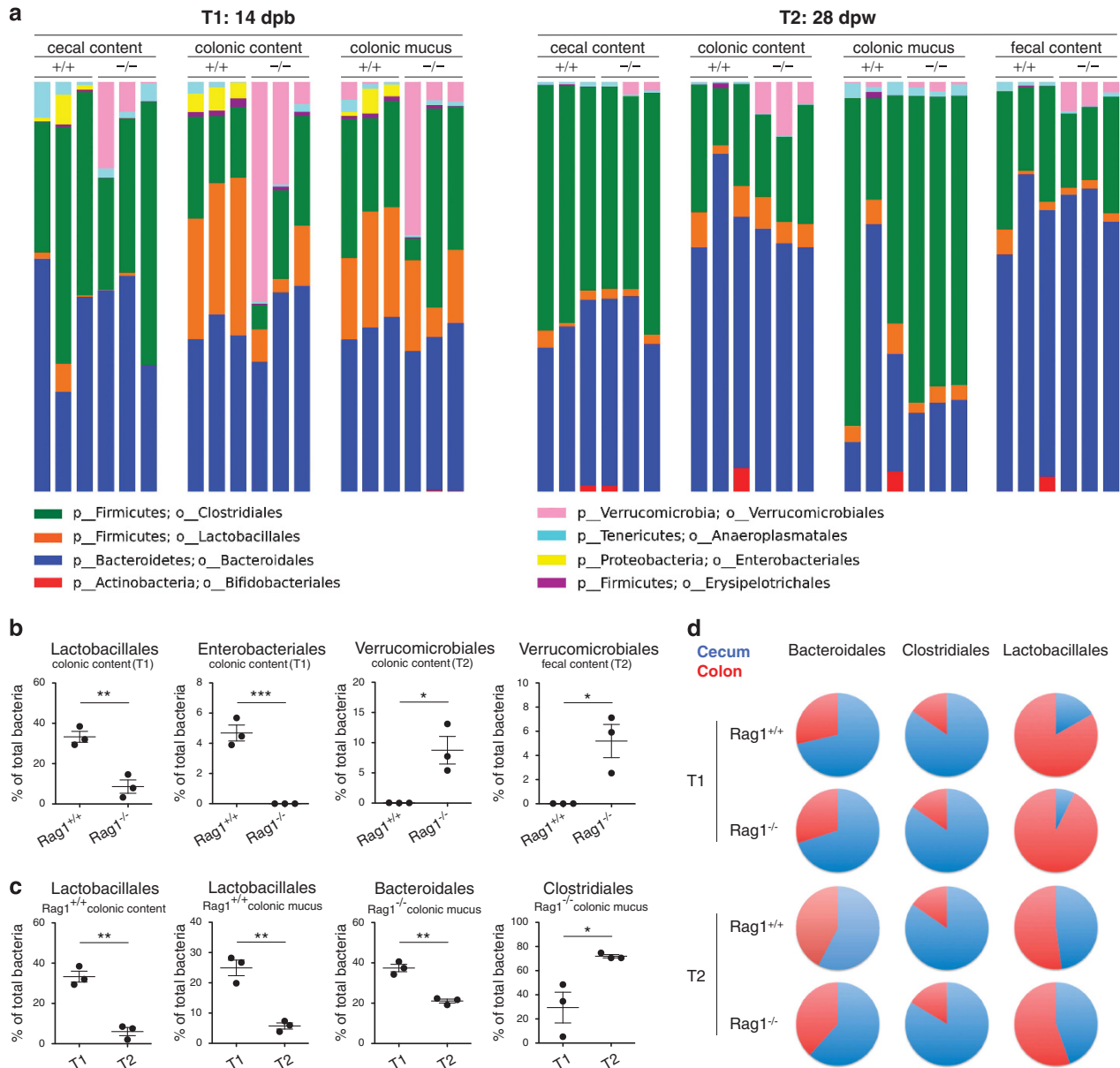


Figure 2 Summary of bacterial taxa for different immunological status, age and anatomical sites. (a) Taxonomic breakdown at the Order level, grouped first by age, then by anatomical sites (cecum, colon, colonic mucus and feces) and finally by Rag1 status (+/+ as Rag1^{+/+} and -/- as Rag1^{-/-}). dpb: days post birth; dpw: days postweaning. Other bacterial taxa accounted for less than 0.1% of total bacteria. (b) Bacterial taxa differentially correlated with Rag mutation. Horizontal lines are plotted as the mean. Error bars are plotted as s.e.m. Statistical comparison was based on unpaired *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (c) Changes of relative abundance from T1 to T2. Statistical comparisons were performed as in b. (d) Proportions of bacterial biomass distributed in cecum versus colon for the three dominant bacterial taxa, Bacteroidales, Clostridiales and Lactobacillales.

Bacteroidales to Clostridiales was noted in colonic mucus of Rag1^{-/-} mice (Figure 2c). The lower level of Bacteroidales in Rag1^{-/-} mice was consistent with the reported higher counts of bacterial flagellin in Rag1^{-/-} mice than wild-type mice (Cullender *et al.*, 2013), as Bacteroidales do not have flagellin (Lozupone *et al.*, 2012). In Rag1^{-/-} mice, a decrease in the abundance of Verrucomicrobiales with age was also observed (Figure 2a) but it was not statistically significant (Supplementary Table S2).

Analysis of different anatomical sites showed distinct distribution patterns of the three abundant

taxa. Based on a weight ratio of 2.3:1 for cecal versus colonic contents (total weight minus wall weight in rodents, estimated according to Pan *et al.* (2009) and Campbell *et al.* (1997)) and relative abundance of taxa, we calculated the bacterial biomass distribution between cecal and colonic contents (Figure 2d). The cecum had consistently higher Bacteroidales and Clostridiales than the colon. However, Lactobacillales, which were more abundant before weaning (Figure 2c), concentrated in the colon (Figure 2d). Immunological status (Rag1^{+/+} or Rag1^{-/-}) had no effect on the anatomical

distribution of the three most abundant taxa. Members of Verrucomicrobiales, on the other hand, were most abundant in the colonic content (Figure 2a and Supplementary Table S3).

Analysis of microbial community diversity

We investigated microbial diversity as a function of immunological status, age and location in the intestine. Microbiota diversity was assessed for both richness (species abundance) and evenness (species distribution). Richness was measured as the number of observed species, phylogenetic diversity (Faith, 1992) and the Shannon index. Evenness was measured by equitability. Rag1^{+/+} and Rag1^{-/-} microbiota had comparable richness and evenness (Figure 3a and Figure 3d). However, all three richness metrics significantly increased with age (Figure 3b). At T1, 331 observed species were identified, and this number increased (30%) to 427 at T2. The phylogenetic diversity, which adds total branch lengths from a site and thus reflects evolutionary divergence of different species, was 28% higher at T2 than T1. The Shannon index also showed the same trend as observed species and phylogenetic diversity. Importantly, we detected an interaction between age and Rag1 status on the Shannon index (two-way analysis of variance, $P=0.028$ for the interaction), which was higher for Rag1^{-/-} than Rag1^{+/+} at T2 but not T1 (Supplementary Figure S2). The evenness also increased with age (Figure 3e). Because equitability (E) and Shannon index (H) are mathematically related ($E=H/\log_2(S_{\text{obs}})$, where S_{obs} is the number of observed species), an interaction between age and Rag1 status was again observed (two-way analysis of variance, $P=0.048$ for the interaction; Supplementary Figure S2). These results suggest that Rag1^{-/-} mice had a higher gain in microbiota diversity with increase in age compared with wild-type mice, possibly due to the lack of control from the adaptive immune system. Interestingly, the colonic mucus had significantly higher richness and evenness than the colonic content and contained the most diverse microbial community among the four anatomical sites (Figures 3c and f).

Regulation of *Akkermansia muciniphila* colonization by adaptive immunity

Because the abundance of Verrucomicrobiales was higher in mice lacking the adaptive immune system (Figures 2a and b and Supplementary Table S1), we decided to further analyze this lineage. In our data set, a total of 117 Verrucomicrobiales-affiliated OTUs were classified at the species level as *A. muciniphila* (Supplementary Figure S3 and Supplementary Table S4), which represented 99.996% of all sequences in the Verrucomicrobia phylum (403 717 sequences affiliated with the 117 OTUs versus a total of 403 733 sequences in the

phylum; the rest 16 sequences were unclassified species in Prosthecobacter (four sequences), Luteolibacter (four sequences), Chthoniobacter (one sequence) or unclassified genera (seven sequences)). The distances between these OTUs were generally less than 5%, as shown in a neighbor-joining phylogenetic tree (Supplementary Figure S4). Importantly, *A. muciniphila* was enriched in Rag1^{-/-} samples compared with significantly lower abundance in Rag1^{+/+} samples (Figure 4a, Mann–Whitney U -test, $P<0.0001$). In order to explicitly investigate the effect of adaptive immunity on *A. muciniphila*, we performed transfer of bone marrow from Rag1^{+/+} to Rag1^{-/-} mice. Adoptive transfer from Rag1^{-/-} to Rag1^{-/-} was also performed as the control. Pre- and post-transfer antibiotics were intentionally avoided while recipient mice were closely monitored. As expected, Rag1^{+/+} bone marrow replenished the spleen and large intestine of Rag1^{-/-} mice with mature T and B cells (Figure 4b). A majority of engrafted T cells were CD4⁺ using the transplantation method. Both early and mature B cells (both B220⁺) were present, as the recipient mice contain endogenous early-stage B cells that are not V(D)J rearranged. As the colonic content had the highest percentage of Verrucomicrobiales in postweaning adult mice (Figure 2a and Supplementary Table S3), colonic contents were collected from recipient mice on which sequencing was performed. A total of 12 samples yielded 48 991 nonchimeric high-quality sequences. As observed in the colonic content of adult Rag1^{+/+} and Rag1^{-/-} mice (Figure 2a), Bacteroidales and Clostridiales dominated colonic microbiota of the recipient mice regardless of the donor bone marrow (Figure 4c). However, the colonic microbiota with Rag1^{+/+} bone marrow transplantation clearly separated from those with Rag1^{-/-} bone marrow transfers on the first principal coordinate PCo1, which explained 34% of variations (Figure 4d). Indeed, the introduction of Rag1^{+/+} bone marrow into Rag1^{-/-} mice significantly decreased the relative abundance of Bifidobacteriales, Bacteroidales and Erysipelotrichales (Supplementary Figure S5). Importantly, the relative abundance of *A. muciniphila* in the colon was repressed more than fivefold in Rag1^{-/-} mice receiving Rag1^{+/+} bone marrow compared with those receiving Rag1^{-/-} bone marrow (Figure 4e), which, together with the observation that the colon of Rag1^{+/+} contained significantly less *A. muciniphila* than that of Rag1^{-/-} mice (Figure 4a), suggests possible control of the colonization of *A. muciniphila* by components of the adaptive immune system that included gut-resident B cells and CD4⁺ T cells.

Discussion

Host–microbe interactions are important for health and disease. How mucosal-colonizing commensal

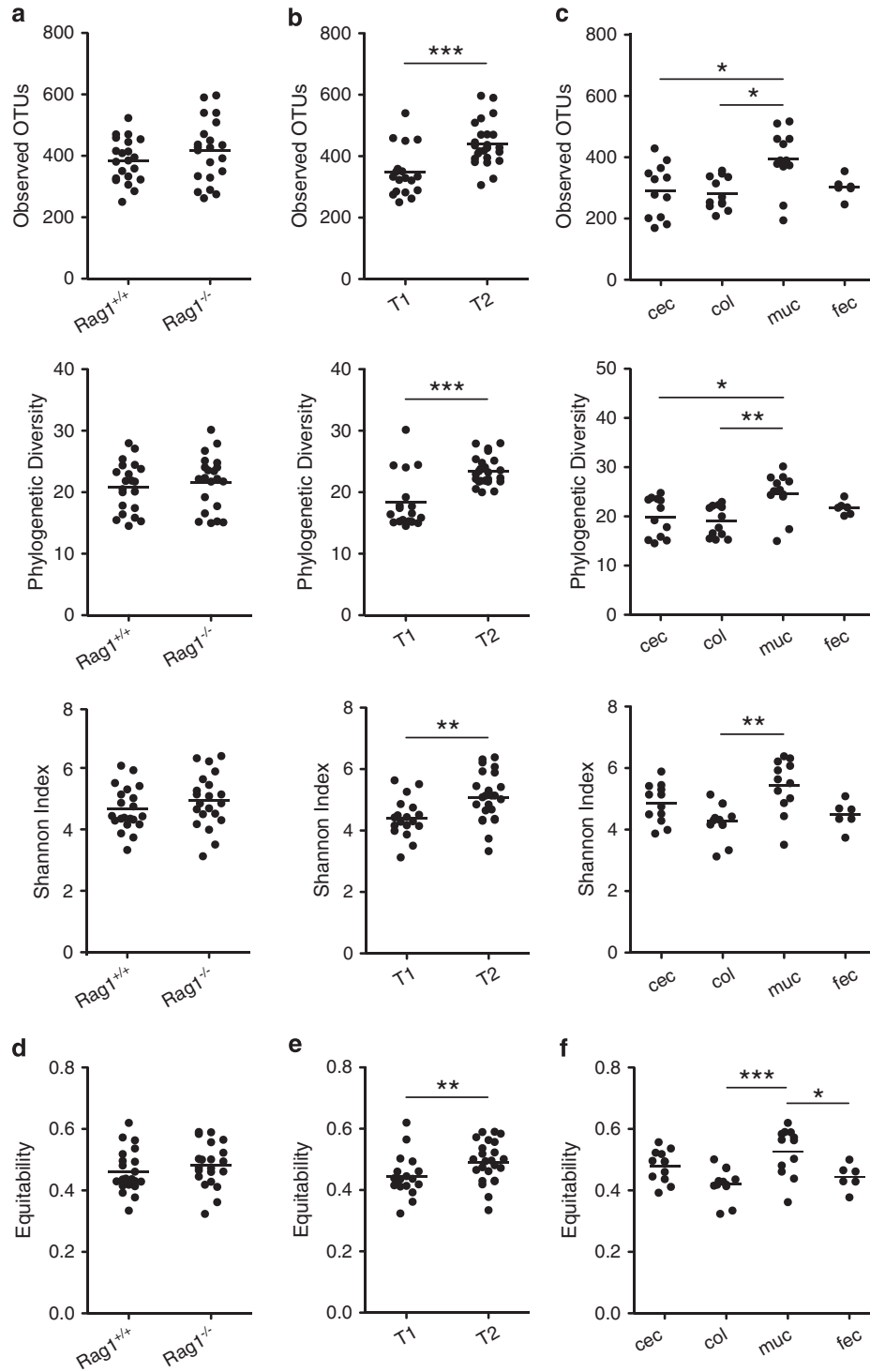


Figure 3 Microbiota community richness and evenness. (a–c) Richness measured as observed OTUs, phylogenetic diversity and Shannon index between microbiota communities from different immunological status (a), age (b) or anatomical site (c). (d–f) Evenness measured as equitability between microbiota communities from different immunological status (d), age (e) or anatomical site (f). Statistical analysis was based on nonparametric Mann–Whitney *U*-test (a/b/d/e) or one-way analysis of variance (c/f). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. cec, cecum; col, colonic content; muc, colonic mucus; fec, feces.

bacteria affect host immunity has been a primary focus of attention. The present study aimed to dissect the opposite function, where host immunity is considered the cause, while microbiota the effect.

Using the classic Rag1^{-/-} model with non-leaky deficiency in adaptive immunity, we showed that the lack of mature lymphocytes led to decreased representation of Lactobacillales and Enterobacteriales

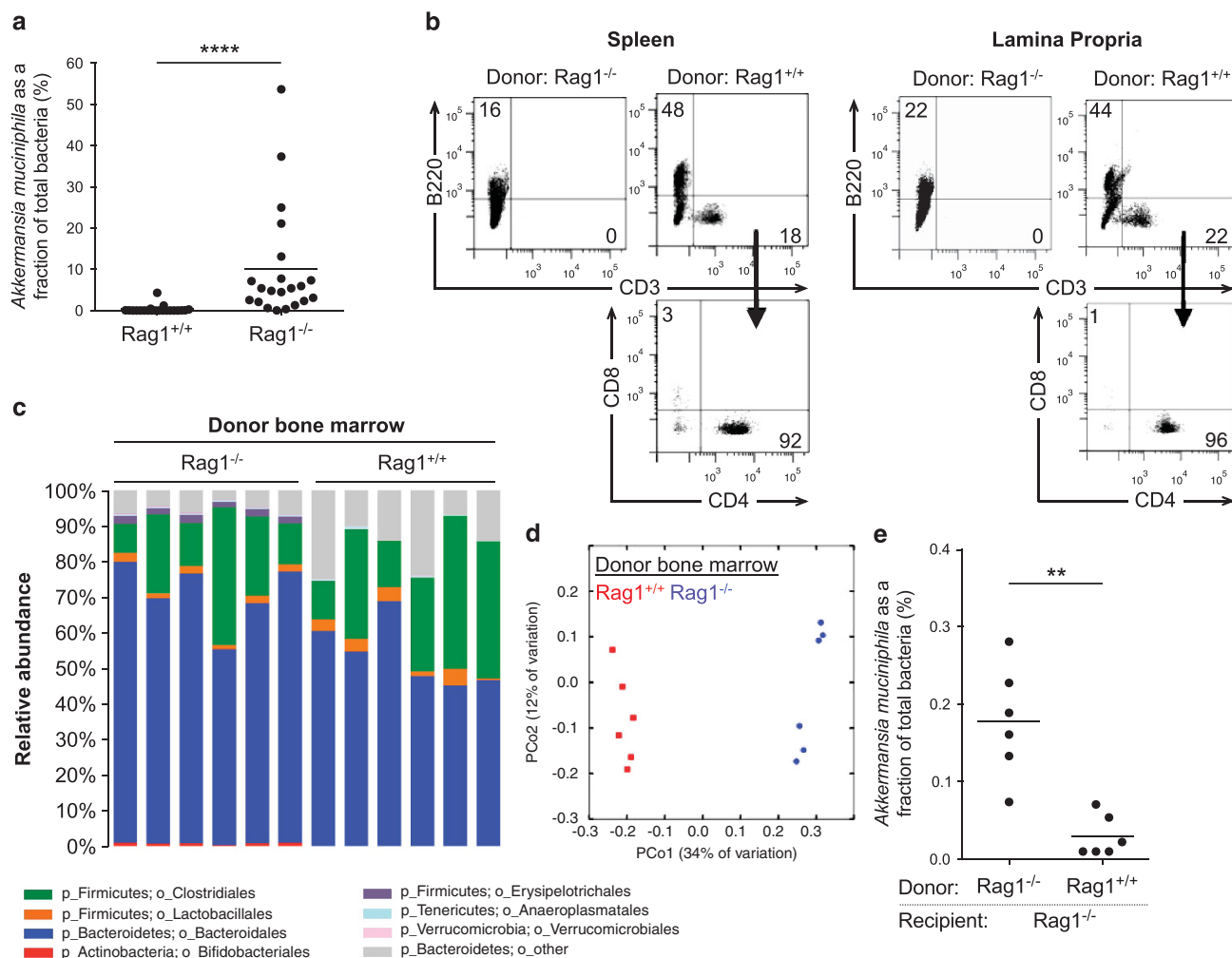


Figure 4 Regulation of *A. muciniphila* colonization by adaptive immunity. **(a)** Increase of *A. muciniphila* with Rag1 deficiency. Statistical significance was obtained with Mann–Whitney *U*-test (**** $P < 0.0001$). **(b–e)** Rag1^{-/-} mice were transplanted with either Rag1^{-/-} ($n = 6$) or Rag1^{+/+} ($n = 6$) bone marrow and analyzed 8 weeks after the transfer. **(b)** Flow cytometry analysis of B and T lymphocytes in the spleen and colonic lamina propria. **(c)** Taxonomic breakdown of all Bacteria in the colon of recipient mice. **(d)** Principal coordinate analysis (PCoA) of mouse colonic microbiota receiving Rag1^{-/-} and Rag1^{+/+} bone marrow transfers, respectively. **(e)** The percentage of *A. muciniphila* in the colon of recipient mice. Statistical comparison was based on unpaired *t*-test (** $P < 0.01$).

in neonates (Figure 2b), and increased amount of Verrucomicrobiales (*A. muciniphila* at the species level) in both neonatal and adult mice (Figure 2a, pink color; Figures 2b and 4a). Analysis of microbial diversity showed that the gut microbiota of wild-type and immunodeficient animals had similar community diversity according to the three metrics compared (Figures 3a and d). In addition, contrary to previous prediction (Willing *et al.*, 2011), the total number of bacteria did not change with immunodeficiency when external factors, such as diet and caging, were carefully controlled (Supplementary Figure S1). Adoptive transfer of Rag1^{+/+} bone marrow, which generated mature B and T cells in the spleen and intestinal lamina propria of post-weaning adult Rag1^{-/-} mice (Figure 4b), reversed the phenotype seen in Rag1^{-/-} mice and suppressed the colonization of *A. muciniphila* in the colon (Figure 4e). Our results suggest that

components of the adaptive immune system can directly alter gut microbiota.

Mice lacking adaptive immunity require different husbandry than wild-type mice, and such difference may directly affect gut microbiota. Several experimental approaches could be used to eliminate the cage effect as a potential confounding factor in our study. We chose to adapt Rag1^{+/+} animals to the same housing conditions as Rag1^{-/-} mice, instead of heterozygous breeding or litter co-housing, to avoid the exposure of Rag1^{-/-} pups to maternal immunoglobulins from Rag1^{+/-} or Rag1^{+/+} dams. Therefore, we described herein the changes of gut microbiota in the absence of the entire adaptive immunity. The effect of maternal immunoglobulins on the microbiota of Rag1^{-/-} mice will be investigated in the future. It has been noted, however, after the completion of this study, that the combination of housing condition adaption and dam co-housing

(prior to breeding) could further equilibrate the baseline microbiota of the two mouse strains. Another feasible approach to eliminate the cage effect could have been to breed heterozygous animals (F0) to generate Rag1^{+/+} and Rag1^{-/-} littermates (F1). Within F1 mice, siblings with the same homozygous genotype could be then mated to generate the wild-type and knockout animals involved in the analysis.

Deficiencies of IgG and IgA, achieved by deleting an enzyme that mediates class switch recombination, were shown to expand segmented filamentous bacteria in the small intestine (Suzuki *et al.*, 2004). However, their effect on the large intestine was not known. A recent study compared adult fecal microbiota of wild-type versus Rag1^{-/-} mice (Dimitriu *et al.*, 2013), but a direct effect of adaptive immunity on microbiota was not demonstrated through adoptive transfer. In addition, whether the microbiota differences between mouse strains were affected by animal age and sampling site was unknown. Using high-throughput sequencing, we have shown a significant lower level of Lactobacillales and complete absence of Enterobacteriales in the colon of neonatal Rag1^{-/-} mice compared with the wild type (Figure 2b). Decreased colonization of these microbes may affect the integrity of gut microbiota as a physical barrier against enteric pathogens. Intriguingly, a consistent increase of *A. muciniphila* was found in the colon of neonatal and adult Rag1^{-/-} mice (Figures 2a, b, and 4a), suggesting that the lack of mature lymphocytes in the intestinal tract and/or the lack of immunoglobulin in the gut and milk may allow the overgrowth of this bacterium. We confirmed adaptive immunity as the cause of this change using an adoptive transfer experiment, where Rag1^{+/+} bone marrow was shown to suppress the colonization of *A. muciniphila* (Figure 4e). The adoptive transfer experiment also excluded the possibility that maternal immunoglobulins were the only cause of microbiota change, as the recipient mice were postweaning adults. Which components of the adaptive immune system could have altered the gut microbiota is yet to be determined. However, by transferring Rag1^{+/+} bone marrow to Rag1^{-/-} mice, we showed that restoration of CD4⁺ T cells and mature B cells in the gut reversed the change of *A. muciniphila* colonization (Figures 4b and e). Whether the gut-resident lymphocytes affect microbiota through producing cytokines or immunoglobulins will be investigated further. Finally, it was noted that the relative abundance of *A. muciniphila* in Rag1^{-/-} mice decreased with age (T1: 0.177 ± 0.060; versus T2: 0.042 ± 0.010; *P* = 0.021). The lower level of *A. muciniphila* in bone marrow transfer experiments (Figure 4e) was likely due to the age effect, as these mice were 14 weeks of age and much older than mice at T1 and T2.

Interestingly, the introduction of Rag1^{+/+} bone marrow into Rag1^{-/-} mice did not appear to

shift the microbiota profile of the recipient mice towards that of Rag1^{+/+} mice (Supplementary Figure S5) except for the relative representation of *A. muciniphila* (Figures 4a and e). The lack of resemblance between the gut microbiota of Rag1^{-/-} mice receiving wild-type bone marrow and that of wild-type mice may be due to the fact that only B cells and CD4⁺ T cells were well engrafted (Figure 4b). Very few CD8⁺ T cells were present in the spleen and colonic lamina propria of reconstituted Rag1^{-/-} mice. In addition, a lower percentage of dendritic cells expressed CD103 in mice receiving Rag1^{+/+} bone marrow than those receiving Rag1^{-/-} bone marrow (Supplementary Figure S6). Both CD103⁺ dendritic cells and CD8⁺ T cells are important for the integrity of intestinal mucosa (Scott *et al.*, 2011; Fleissner *et al.*, 2010). Therefore, the microbiota changes observed in the bone marrow transplantation experiment may pertain to the effects of B cells and/or CD4⁺ T cells, and not CD8⁺ T cells or CD103⁺ dendritic cells.

The Gram-negative bacterium *A. muciniphila* has been recently found to be enriched following antibiotics treatment (Dubourg *et al.*, 2013; Hansen *et al.*, 2013) and have the capability to control inflammation (Everard *et al.*, 2013). Initially considered as a host mucin-degrading bacterium, *A. muciniphila* was found to colonize the mouse gut without consuming much host-derived compounds (Berry *et al.*, 2013). Although the physiological function of this bacterium in immunocompromised hosts is still unclear, our results suggest that *A. muciniphila* may be used as a biomarker for immunodeficiency. Identification of such biomarker is especially valuable for infants with severe combined immunodeficiency (SCID), commonly known as the 'bubble boy disease'. Young children without a family history of SCID are often not diagnosed until 6 months old or older. If untreated, the disease is almost always fatal within the first year of age. In addition, immunodeficient infants should not receive live virus vaccinations. Therefore, early diagnosis of SCID, potentially through measuring the abundance of *A. muciniphila* in the feces of infants not treated with antibiotics, can be beneficial. Whether or not immunodeficient babies indeed have *A. muciniphila* enriched in their feces will be determined in the future.

Our results also showed that distal gut microbiota's community structure (Figure 1c) and microbial diversity (Figures 3b and e) differed with animal age. As young pups started to consume solid food, the abundance of Lactobacillales decreased (Figure 2c). In Rag1^{-/-} animals, more Clostridiales and fewer Bacteroidales were identified postweaning. This observation is consistent with the previous report on the expansion of Clostridiales-related segmented filamentous bacteria in mice lacking IgA (Suzuki *et al.*, 2004). We also observed that microbial diversity increased with age (Figures 3b and e), a finding that is

consistent with a recent 2.5-year longitudinal monitoring of human infant microbiota (Koenig *et al.*, 2010). Despite the high number of sequences obtained in this study and others, the number of OTUs usually does not reach a plateau, indicating that microbiota have been under-sampled. Thus, the increased diversity at T2 was likely a result of increased representation of rare OTUs that were below the detection limit at T1 but became detectable at T2. It cannot be ruled out, however, that the increase of microbial diversity might have come from acquisition of bacteria in the dust and aerosols as the housing environment was not completely germ-free.

Interestingly, we found that among our four sampling sites, colonic mucus contained the highest microbial diversity (Figures 3c and f). The outer layer of colonic mucus is known to harbor commensal bacteria (Johansson *et al.*, 2008). The higher observed mucosal microbial diversity in our study are likely to be an effect of multiple factors, among which, the availability of renewable mucin glycoprotein reduced axial movement compared with the lumen (Deplancke and Gaskins, 2001), and increased colonization of aerobic bacteria that can utilize oxygen diffused from the tissues surrounding the intestinal lumen (Pedron *et al.*, 2012). Furthermore, we found that Rag1^{-/-} mice had a higher gain in microbiota richness and evenness with increase in age compared with wild-type mice (Supplementary Figure S2), possibly due to the lack of control from the adaptive immune system.

Taken together, we provided evidence that adaptive immunity could alter the composition and diversity of gut microbiota. Defects of adaptive immune system, such as in primary immunodeficiency (for example, SCID) or acquired immunodeficiency (for example, HIV infection), may change gut microbiota that would in turn modulate the immune response to deteriorate or compensate for the defects. We identified in this study a potential fecal biomarker for early diagnosis of infantile SCID. An increased level of fecal *A. muciniphila*, which normally exists at a negligible level in the feces of healthy young children (Costello *et al.*, 2013), may prompt timely examinations and potentially prevent pediatric emergencies associated with SCID.

Conflict of Interest

The authors declare no conflict of interest.

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