AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VIRUS-CELL INTERACTIONS

# Subclinical Cytomegalovirus Infection Is Associated with Altered Host Immunity, Gut Microbiota, and Vaccine Responses

Clarissa Santos Rocha,[a] Lauren A. Hirao,[a] Mariana G. Weber,[a] Gema Méndez-Lagares,[a,b] W. L. William Chang,[c] Guochun Jiang,[a] Jesse D. Deere,[c] Ellen E. Sparger,[a] Jeffrey Roberts,[b] Peter A. Barry,[b,c,d] Dennis J. Hartigan-O'Connor,[a,b] Satya Dandekar[a,b]

[a]Department of Medical Microbiology and Immunology, University of California, Davis, California, USA
[b]California National Primate Research Center, Davis, California, USA
[c]Center for Comparative Medicine, Davis, California, USA
[d]Department of Pathology and Laboratory Medicine, Davis, California, USA

**ABSTRACT** Subclinical viral infections (SVI), including cytomegalovirus (CMV), are highly prevalent in humans, resulting in lifelong persistence. However, the impact of SVI on the interplay between the host immunity and gut microbiota in the context of environmental exposures is not well defined. We utilized the preclinical nonhuman primate (NHP) model consisting of SVI-free (specific-pathogen-free [SPF]) rhesus macaques and compared them to the animals with SVI (non-SPF) acquired through natural exposure and investigated the impact of SVI on immune cell distribution and function, as well as on gut microbiota. These changes were examined in animals housed in the outdoor environment compared to the controlled indoor environment. We report that SVI are associated with altered immune cell subsets and gut microbiota composition in animals housed in the outdoor environment. Non-SPF animals harbored a higher proportion of potential butyrate-producing *Firmicutes* and higher numbers of lymphocytes, effector T cells, and cytokine-producing T cells. Surprisingly, these differences diminished following their transfer to the controlled indoor environment, suggesting that non-SPFs had increased responsiveness to environmental exposures. An experimental infection of indoor SPF animals with CMV resulted in an increased abundance of butyrate-producing bacteria, validating that CMV enhanced colonization of butyrate-producing commensals. Finally, non-SPF animals displayed lower antibody responses to influenza vaccination compared to SPF animals. Our data show that subclinical CMV infection heightens host immunity and gut microbiota changes in response to environmental exposures. This may contribute to the heterogeneity in host immune response to vaccines and environmental stimuli at the population level.

**IMPORTANCE** Humans harbor several latent viruses that modulate host immunity and commensal microbiota, thus introducing heterogeneity in their responses to pathogens, vaccines, and environmental exposures. Most of our understanding of the effect of CMV on the immune system is based on studies of children acquiring CMV or of immunocompromised humans with acute or reactivated CMV infection or in ageing individuals. The experimental mouse models are genetically inbred and are completely adapted to the indoor laboratory environment. In contrast, nonhuman primates are genetically outbred and are raised in the outdoor environment. Our study is the first to report the impact of long-term subclinical CMV infection on host immunity and gut microbiota, which is evident only in the outdoor environment but not in the indoor environment. The significance of this study is in highlighting the impact of SVI on enhancing host immune susceptibility to environmental exposures and immune heterogeneity.

**KEYWORDS** CMV, gut microbiota, immunity, cytomegalovirus

**Received** 30 January 2018 **Accepted** 14 April 2018

**Accepted manuscript posted online** 18 April 2018

**Citation** Santos Rocha C, Hirao LA, Weber MG, Méndez-Lagares G, Chang WLW, Jiang G, Deere JD, Sparger EE, Roberts J, Barry PA, Hartigan-O'Connor DJ, Dandekar S. 2018. Subclinical cytomegalovirus infection is associated with altered host immunity, gut microbiota, and vaccine responses. J Virol 92:e00167-18. https://doi.org/10.1128/JVI.00167-18.

**Editor** Jae U. Jung, University of Southern California



Address correspondence to Satya Dandekar, sdandekar@ucdavis.edu.

C.S.R. and L.A.H. contributed equally to this article.

There is a substantial diversity in the immune status of humans at the population level that contributes to variable immune responses to vaccines and therapy (1). Mechanisms driving this immune variation are not fully understood. Humans harbor several latent viruses, including cytomegalovirus (CMV) that are implicated in the modulation of host immunity (2). However, the impact of subclinical viral inections (SVI) on the interplay between the host immunity and gut microbiota in the context of environmental exposures is not well defined.

Although contributions of age, diet, and gut microbiota on host immune status are being well investigated (3, 4), there is limited understanding of the influence of lifelong persistent latent viral infections, most notably of the herpesviruses, including CMV and Epstein-Barr virus (EBV). The seropositivity of CMV among adults is 90% in Africa and Asia or 60 to 70% in the United States and Europe (5). While the majority of CMV-infected individuals are asymptomatic due to effective immune control, reactivation of the virus in immunosuppressed individuals can cause clinical illness (6). About 10% of memory T cells are found to be CMV-specific in healthy adults with long-term subclinical CMV infection, suggesting interactions between the host immune system and the virus (7). The maintenance of the high virus-specific T cell responses, coupled with an age-related expansion, are likely related to the viral antigenic exposure due to the recurrent viral reactivation (8). This rigorous immune surveillance is reflected by an increase of $CD8^+$ T cells and B cells, and a decrease in the naive $CD8^+$ T cell population (9). Whether these CMV-induced immune changes might modulate host responses to environmental exposures or alter gut microbiota is unknown. Previous studies examining the effects of CMV seropositivity on human immune responses to influenza virus vaccination have yielded different outcomes (10–14).

The gut microbiota have been shown to modulate the development and functioning of the host immune system through production of small molecules including short-chain fatty acids (SCFAs), which stimulate differentiation of immune cells and epithelial cells and promote host immunity (15). Gut microbiota also support the induction of effective host vaccine responses through TLR5 (16), trigger antiviral interferon (IFN) responses against pathogens (17), and maintain memory cell populations in viral infections (18). Microbial dysbiosis is well documented in symptomatic viral infections and in noninfectious inflammatory diseases (19–21). In HIV and simian immunodeficiency virus (SIV) infections, correlations between microbial dysbiosis, aberrant pathogen recognition receptor signaling, and inflammatory cytokine production in the gut were observed and, importantly, were linked to chronic immune activation (22, 23). However, there is a lack of information regarding the impact of SVI, such as CMV, on the gut microbiota and associated host immunity and whether these changes are further modulated due to environmental exposures in the outdoor environment.

Human immune complexity is not adequately recapitulated in laboratory mouse models since they are genetically inbred and housed in extremely hygienic indoor environment and often fail to predict efficacy of vaccines and therapeutics in human populations (24, 25). In contrast, rhesus macaques are genetically outbred and share a significant genetic homology, as well as common physiological and immunological characteristics with humans (26). To investigate the role of SVI in host immunity and gut microbiota complexity, we utilized a preclinical translational nonhuman primate (NHP) model, which consisted of specific-pathogen-free (SPF) rhesus macaques and age-matched non-SPF animals. The SPF animals are free of persistent latent viral infections, including infections with rhesus cytomegalovirus (RhCMV), simian B virus, simian foamy virus (SFV), and simian beta retrovirus (SRV). In contrast, non-SPF animals are naturally exposed to viral infections endemic in breeding cohorts of NHPs, including RhCMV and other herpesviruses. Importantly, both SPF and non-SPF animals are housed in the outdoor setting and experience similar environmental exposures. Our study tested the hypothesis that subclinical CMV infection may accentuate changes in the host immunity and gut microbiota in the context of environmental exposures. Investigation of gut microbiota and immune cells from the SPF and non-SPF animals showed that subclinical CMV infection was associated with marked differences in immune cell populations

and gut microbiota composition in animals that were housed in the outdoor environment. Non-SPF animals showed greater numbers of lymphocytes, effector T cells, cytokine-producing T cells, and an increased abundance of potential SCFA-producing bacteria, especially butyrate-producing microbes. An experimental CMV infection of SPF animals clearly demonstrated an increase in the abundance of potential butyrate-producing gut microbiota. Surprisingly, these differences between SPF and non-SPF animals in immune responses and gut microbiota diminished substantially when animals were housed in the built-in indoor environment. Despite the minimal differences observed in the indoor environment, immunization with an influenza vaccine resulted in significantly greater levels of influenza-specific antibody response in the SPF compared to non-SPF animals in the indoor environment, linking the difference in vaccine response to the presence of subclinical viruses. Our data highlight the impact of SVI on changes in the host immune cells and gut microbiota in the context of indoor and outdoor environmental exposures. This may explain why several vaccine and therapeutic studies performed on indoor housed animals may not fully predict immune variation observed in the immune responses among humans at the population level. In summary, our data show that subclinical CMV infection may accentuate immune variation by modulating the host immune system and gut microbiota in response to environmental exposures.

## RESULTS

**Marked differences in the gut microbiota of animals from an outdoor environment versus an indoor environment.** Nonhuman primates at the California National Primate Research Center (CNPRC) are housed in an outdoor environment. Animals are moved to the built indoor environment for experimental studies and sample collections. We sought to determine whether housing the animals in an outdoor environment compared to a controlled indoor environment had any effect on their gut microbiota. It is noteworthy that outdoor animals are exposed to different environmental antigens and microbes, while the animals housed in the controlled indoor environment have limited such exposures. However, they may experience several stressors, including social disruption and limited exposure to the natural daylight and exercise. Although animals in outdoor and indoor environments receive the same diet, it is possible that outdoor animals might be ingesting different plant-derived compounds.

The gut microbiota of outdoor animals clustered distinctly from the gut microbiota of indoor animals ($P = 0.001$, PERMANOVA [permutational multivariate analysis of variance] test) as shown by unweighted UniFrac principal coordinate analysis (PCoA) (Fig. 1A). The most abundant families in the outdoor animals consisted of *Prevotellaceae*, *Helicobacteraceae*, *Ruminococcaceae*, *Paraprevotellaceae*, and *Veillonellaceae*, while the indoor animals had a high prevalence of *Prevotellaceae*, *Ruminococcaceae*, p-2534-18B5, *Spirochaetaceae*, and *Paraprevotellaceae* (Fig. 1B). We performed linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify bacterial taxa within the three most abundant phyla (*Firmicutes*, *Bacteroidetes*, and *Proteobacteria*) that were characteristic of the animals housed outdoors versus indoors. Overall, *Firmicutes* were highly enriched in the indoor animals. Several members of the *Clostridia* class, including *Ruminococcaceae*, *Christensenellaceae*, *Streptococcus*, and *Oscillospira*, were overly represented in indoor animals compared to outdoor animals. In contrast, only three members of *Firmicutes*, including *Faecalibacterium* and *Anaerovibrio*, were associated with outdoor animals. Members of the phyla *Bacteroidetes* and *Proteobacteria*, including *Prevotella*, *Sutterella*, *Alcaligenaceae*, and *Campylobacter*, were found predominantly in animals housed outdoors (Fig. 1C). Importantly, we did not observe any significant effect of age and sex on the microbiota profiles in animals from both indoor and outdoor environments (see Fig. S1A and B in the supplemental material).

To define the functional capacity of the gut microbiota from indoor and outdoor animals, we examined potential metagenomic contributions of the microbial community by using PICRUSt (27). The LEfSe analysis of the predicted KEGG metabolic

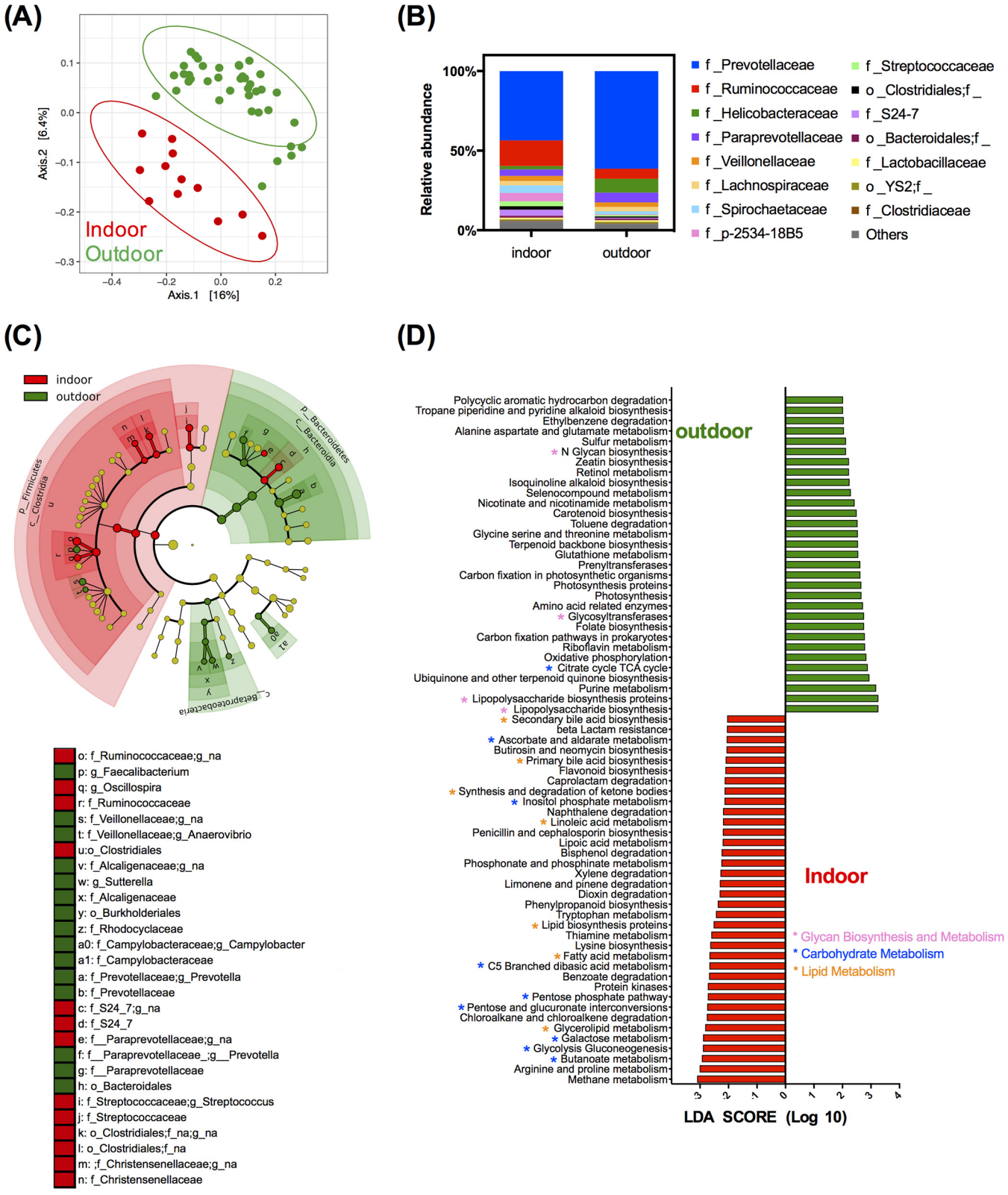


**FIG 1** Differences in the composition and functionality of the gut microbiota of rhesus macaques from outdoor and indoor environments. (A) Unweighted UniFrac PCoA analysis showed a marked difference in gut microbiota composition of animals from outdoor versus indoor environment. (B) Bar charts are showing the relative abundance of bacterial families among animals from the outdoor and indoor environments. (C) Cladogram representing the taxonomic hierarchical structure of bacterial taxa generated by LEfSe analysis. Bacterial taxa enriched in indoor animals are represented in red, and those from the outdoor environment are represented in green. The yellow circles represent bacterial taxa that were not significantly changed. The analyses were restricted to the three most abundant phyla (*Firmicutes*, *Bacteroidetes*, and *Proteobacteria*). Selection of discriminative taxa between the groups was

(Continued on next page)

pathways highlighted enrichment of 36 pathways in indoor animals and 31 pathways in outdoor animals (Fig. 1D). Notably, carbohydrate metabolism pathways had an increased representation in indoor animals compared to outdoor animals (22% versus 3%). Lipid metabolism pathways were exclusively associated with indoor animals (19% of the total pathways identified in indoor animals), and glycan metabolism was exclusively associated with outdoor animals (13% of the total pathways identified in outdoor animals) (Fig. 1D). Collectively, our data show that environmental factors play an important role in shaping the microbiota composition and functionality of rhesus macaques.

**Subclinical viral infections accentuate differences in the gut microbiota composition only in the outdoor but not in the indoor environment.** SVI are prevalent among animals and humans and may influence the commensal microbiota of infected hosts. We examined whether SVI influenced changes in the gut microbial communities from rhesus macaques in the outdoor environment in comparison to the indoor environment.

No significant differences were detected in the beta diversity of the gut microbiota between SPF and non-SPF animals housed either outdoors or indoors, as shown by unweighted UniFrac PCoA analysis, indicating that subclinical viral infections do not significantly alter the overall composition of the gut microbiome (Fig. 2A). However, direct comparisons of microbial communities at all taxonomic levels between outdoor-housed SPF and non-SPF animals highlighted 12 bacterial taxa that were significantly different and predominantly mapped to butyrate-producing bacteria within the *Firmicutes* phylum (see Table S1 in the supplemental material). Differences between groups were considered statistically significant when they met the criteria of $P \leq 0.05$ and false discovery rate (FDR)-corrected $P$ (q-value) $\leq 0.1$ The abundances of *Lachnospiraceae*, *Clostridiaceae*, *Faecalibacterium*, *Roseburia*, *Clostridium*, and *Coprococcus* were significantly increased in non-SPF animals. The abundance of *Phascolarctobacterium*, a propionate producer, was also increased in non-SPF animals (28) (Fig. 2B). In non-SPF animals, highly abundant butyrate-producing bacteria included members of *Clostridium* groups XIVa, XI, and IV. These bacteria are known to be the major butyrate producers in the gastrointestinal tract (29).

Evaluation of the gut microbiota profiles of SPF and non-SPF animals housed in the indoor controlled environment showed that their gut microbiota profiles were very similar. It was surprising to note that the microbiota differences observed between SPF and non-SPF animals in the outdoor setting diminished when the animals were housed indoors. The prevalence of potential SCFA-producing microbes was similar in the SPF and non-SPF animals in the indoor environment (Fig. 2C).

We sought to examine whether the presence of SVI was associated with changes in the functional capacity of the gut microbiota in non-SPF animals. LEfSe analysis of the metabolic pathways highlighted the enrichment of 12 KEGG pathways in non-SPF and 8 pathways in SPF macaques in the indoor environment. Pathways related to lipid metabolism (ether lipid, glycerolipid, and primary and secondary bile acid synthesis) and the biosynthesis of secondary metabolites (flavone, flavonol, and phenylpropanoid) were exclusively associated with non-SPF (RhCMV-seropositive) animals. In contrast, N-glycan biosynthesis and metabolism of vitamins and cofactors were associated only with SPF animals (Fig. 2D). Importantly, analysis of individual KOs predicted by PICRUSt revealed an increased enrichment of butyryl coenzyme A (butyryl-CoA) dehydrogenase gene in non-SPF animals compared to SPF animals (Fig. 2E). This is a key enzyme involved in the butyrate synthesis pathway (30). The LEfSe analysis of the predicted KEGG metabolic pathways was performed for the indoor animals; no specific pathways were associated with either SPF or non-SPF animals, suggesting that gut

**FIG 1** Legend (Continued)
based on an LDA score cutoff of 2.0. na, not assigned. (D) LEfSe analyses of KEGG metabolic pathways were predicted by PICRUSt. Pathways with a positive LDA score (green) are enriched in outdoor animals and pathways with a negative LDA score (red) are associated with indoor animals (indoor animals, $n = 12$; outdoor animals, $n = 40$).

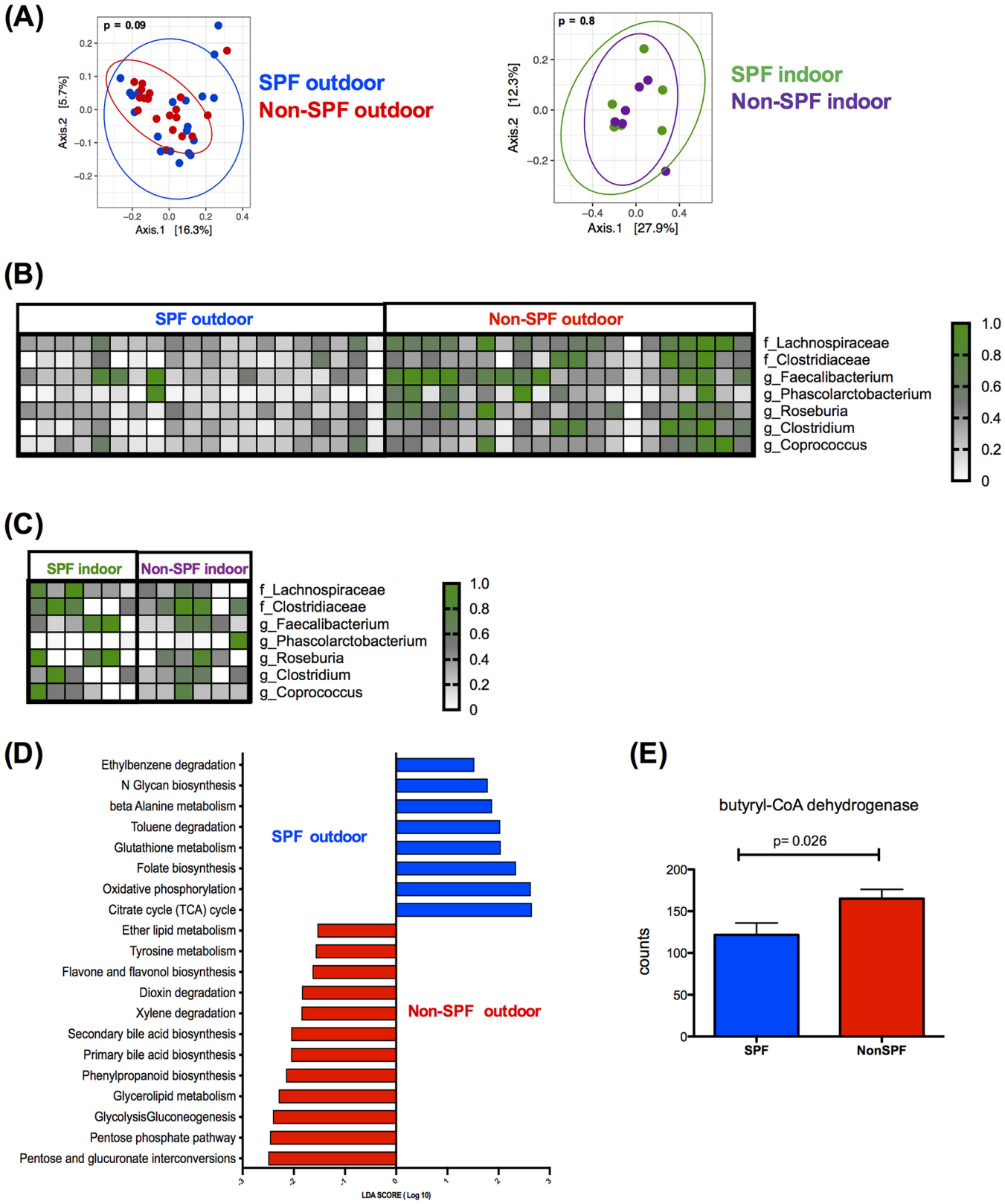


**FIG 2** Subclinical viral infections increase abundance of butyrate-producing microbes only in the outdoor environment. (A) No significant differences were detected in the overall gut microbiota composition of SPF and non-SPF animals housed either in the outdoor or indoor environment as detected by beta diversity (unweighted UniFrac). (B) The non-SPF animals have an increased abundance of butyrate producers compared to SPF animals in the outdoor environment. The heatmap shows scaled values for the relative abundance of potential butyrate producers detected in SPF and non-SPF animals. Each column represents a different animal. Only bacteria taxa statistically different between SPF and non-SPF ($P \leq 0.05$ and q-value $\leq 0.1$) are shown. (C) No significant

(Continued on next page)

microbiota from indoor animals have a similar functional capacity that is independent of the presence of subclinical viral infections. In summary, our data show that persistent subclinical viral infections are linked to changes in the composition, as well as the metabolism, of the gut microbiota in non-SPF animals housed in the outdoor environment but not in the indoor setting.

**Experimental CMV infection of SPF macaques resulted in increased prevalence of butyrate-producing microbes.** We sought to investigate the role of CMV in inducing changes in the gut microbiota of the host because we observed an increased abundance of potential SCFA-producing bacteria in non-SPF animals only in the outdoor environment. Four indoor SPF animals were experimentally infected with RhCMV and monitored for changes in the frequency of potential butyrate and propionate producers following the primary viral infection. All animals were successfully infected with the virus as evidenced by CMV seropositivity (Fig. 3A). We found that the levels of many SCFA-producing bacteria markedly increased following experimental CMV infection and remained elevated for at least 16 weeks (Fig. 3B). At 2 weeks postinfection, significant increases in abundance of *Coprococcus* (fold change [FC] = 5.6, $P = 0.03$) and *Faecalibacterium* (FC = 3.2, $P = 0.03$) were observed. At 4 weeks postinfection, *Clostridiaceae* (FC = 5.1, $P = 0.003$) was significantly elevated compared to the preinfection baseline values (Fig. 3B). We also monitored the population of potential SCFA producers in SPF animals that served as CMV-negative controls. We did not observe any significant changes over time in the gut microbiota (Fig. 3C), demonstrating that CMV infection leads to an increased colonization of potential butyrate-producing microbes.

**Increased prevalence of butyrate-producing microbes in non-SPF animals correlates with RhCMV-specific antibody levels in the outdoor environment.** Previous studies reported that SCFAs, including butyrate and propionate, can inhibit histone deacetylase and thereby activate latent CMV and EBV (31, 32). Activation of HIV transcription by butyrate has also been reported (33). To determine whether differences in the butyrate-producing gut microbial communities during subclinical viral infections are linked to CMV reactivation, we examined correlations between the presence of the most abundant bacteria taxa at the lowest taxonomic level (>0.1% in all samples) and the levels of RhCMV-specific antibodies. It has been previously well established that the levels of RhCMV-specific antibodies correlate with RhCMV shedding (34). Although we did not directly determine CMV replication, we measured CMV-specific antibody levels as an indicator of CMV shedding in the host.

Overall, the abundance of SCFA-producing bacteria, especially butyrate producers, positively correlated with the levels of RhCMV antibody in non-SPF animals, suggesting a frequent reactivation of latent CMV (Fig. 4A). The abundance of *Faecalibacterium* (rho = 0.47, $P = 0.04$), *Lachnospiraceae* (rho = 0.45, $P = 0.04$), and *Phascolarctobacterium* (rho = 0.53, $P = 0.02$) exhibited significant positive correlations with RhCMV antibody levels (Fig. 4B).

**Increased levels of lymphocytes and effector T cells in non-SPF animals compared to SPF animals in the outdoor environment.** To determine the impact of subclinical viral infections on immune cell subsets and function, immunophenotypic analysis was performed on peripheral blood mononuclear cells (PBMC) from SPF and non-SPF rhesus macaques housed outdoors.

Remarkably, non-SPF animals had 3-fold-higher numbers of lymphocytes compared to SPF animals (Fig. 5A). In multiple regression analysis, this difference did not correlate with the age or sex of these animals. Measurement of the frequencies of naive, effector,

**FIG 2** Legend (Continued)
differences in the abundance of butyrate producers were found between SPF and non-SPF animals in the indoor environment. (D) LEfSe analyses of KEGG metabolic pathways predicted by PICRUSt. Pathways with a positive LDA score (blue) are enriched in SPF animals, and pathways with a negative LDA score (red) are associated with non-SPF animals. (E) Butyryl-CoA dehydrogenase gene counts predicted by PICRUSt in outdoor animals. Increased prevalence of butyryl-CoA dehydrogenase gene was found in non-SPF animals (35% increase) compared to SPF animals ($P < 0.05$, two-tailed Mann-Whitney test). Data are shown as group means ± the standard errors of the mean (SEM). SPF outdoor, $n = 20$; non-SPF outdoor, $n = 20$; SPF indoor, $n = 6$; non-SPF indoor, $n = 6$.

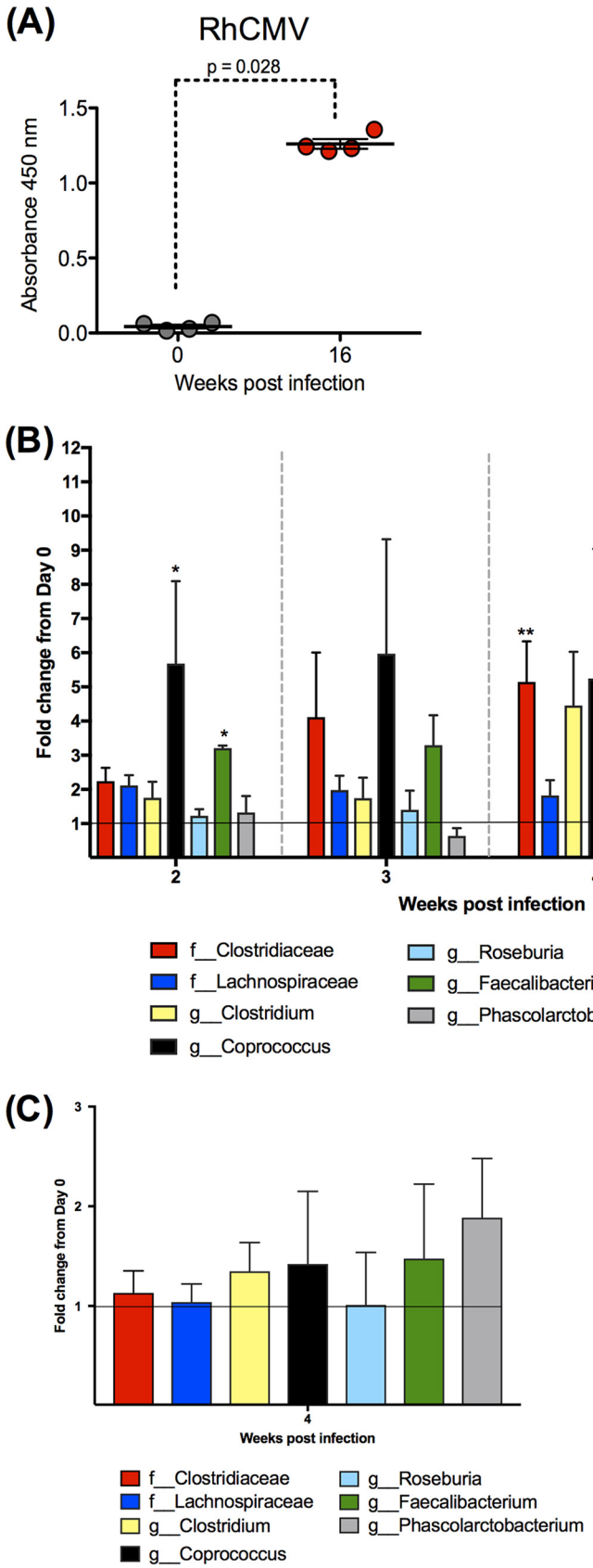


**FIG 3** Experimental CMV infection of indoor SPF macaques led to increased abundance of potential butyrate-producing microbes. SPF animals ($n$ = 4) were experimentally infected with RhCMV, and the frequencies of

(Continued on next page)

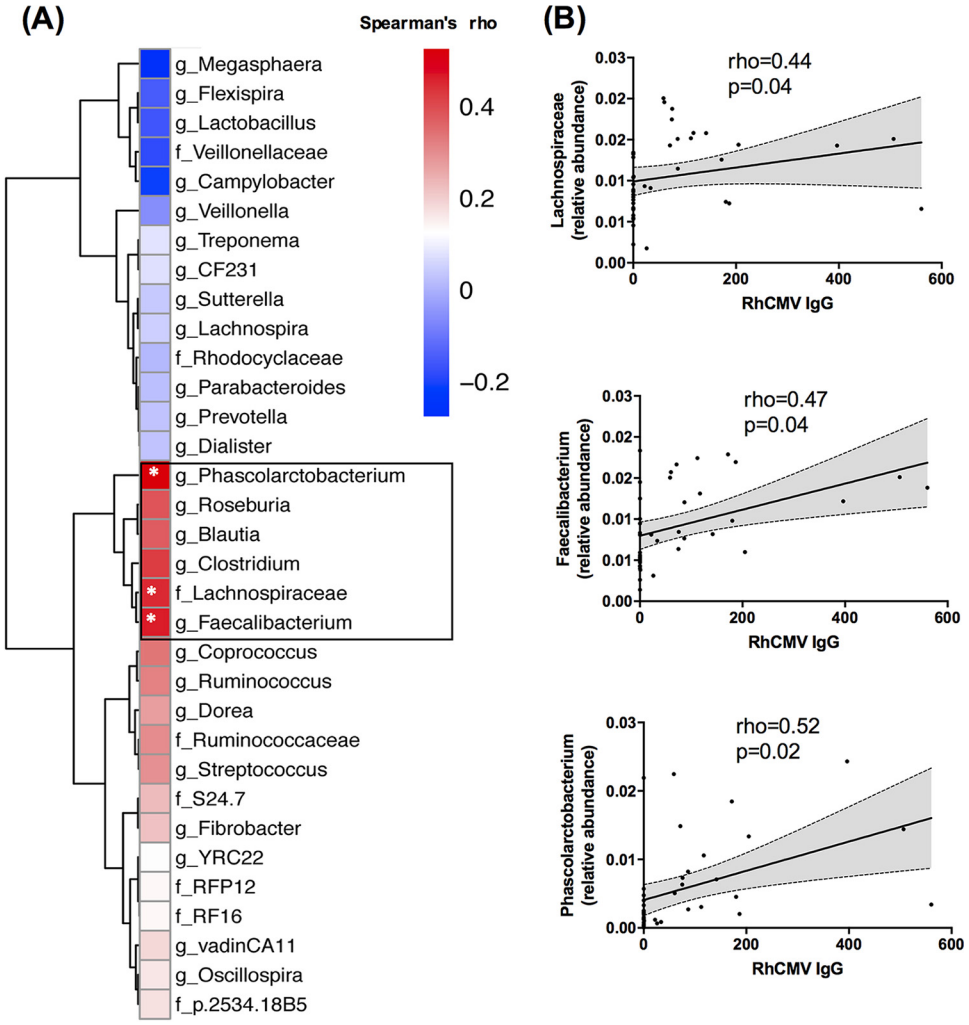


**FIG 4** Correlation between the abundance of SCFA-producing microbes with RhCMV-specific antibody levels in the animals from outdoor environment. (A) Heatmap of Spearman correlation coefficients between the most abundant bacteria taxa at the lowest taxonomic level (>0.1% in all samples) and RhCMV-specific antibody levels. The red color indicates a positive correlation, and the blue color represents a negative correlation. Significant correlations (FDR-adjusted $P < 0.05$) are indicated with an asterisk. (B) The abundance of *Lachnospiraceae*, *Faecalibacterium*, and *Phascolarctobacterium* significantly positively correlated with RhCMV-specific antibody titers.

and memory CD8$^+$ T cell populations in SPF and non-SPF animals by flow cytometry revealed that non-SPF animals had significantly higher levels of effector (CD28$^-$ CD95$^+$) and lower levels of memory (CD28$^+$ CD95$^+$) CD8$^+$ T cells compared to SPF animals (Fig. 5B; see reference 2). As for the CD4$^+$ T cell populations in SPF and non-SPF animals, there were no major differences in the distribution of effector and memory cells or proliferating CD4$^+$ T cells (see Fig. S2A and B in the supplemental material). Functional T-cell responses to heat-inactivated RhCMV virions showed the presence of

**FIG 3** Legend (Continued)
butyrate and propionate producers were monitored during the course of CMV infection. (A) RhCMV-specific antibodies were detected by ELISA in plasma samples at 16 weeks postinfection as a measure of CMV infection. (B) Abundance of SCFA-producing microbes increased following experimental CMV infection. Significant increase in *Coprococcus* and *Faecalibacterium* was observed at 2 weeks postinfection and *Clostridiaceae* was significantly elevated at 4 weeks postinfection compared to preinfection baseline values. Friedman, followed by Dunn's posttest, was used for comparisons of gut microbiota changes over time following the experimental RhCMV infection of indoor SPF animals. Significant differences are represented by asterisks (*, $P < 0.05$; **, $P < 0.01$). (C) SPF animals ($n = 5$) without RhCMV infection served as controls and were monitored for changes in the population of potential SCFA producers over time; no significant changes were observed (Wilcoxon matched-pair signed-rank test).

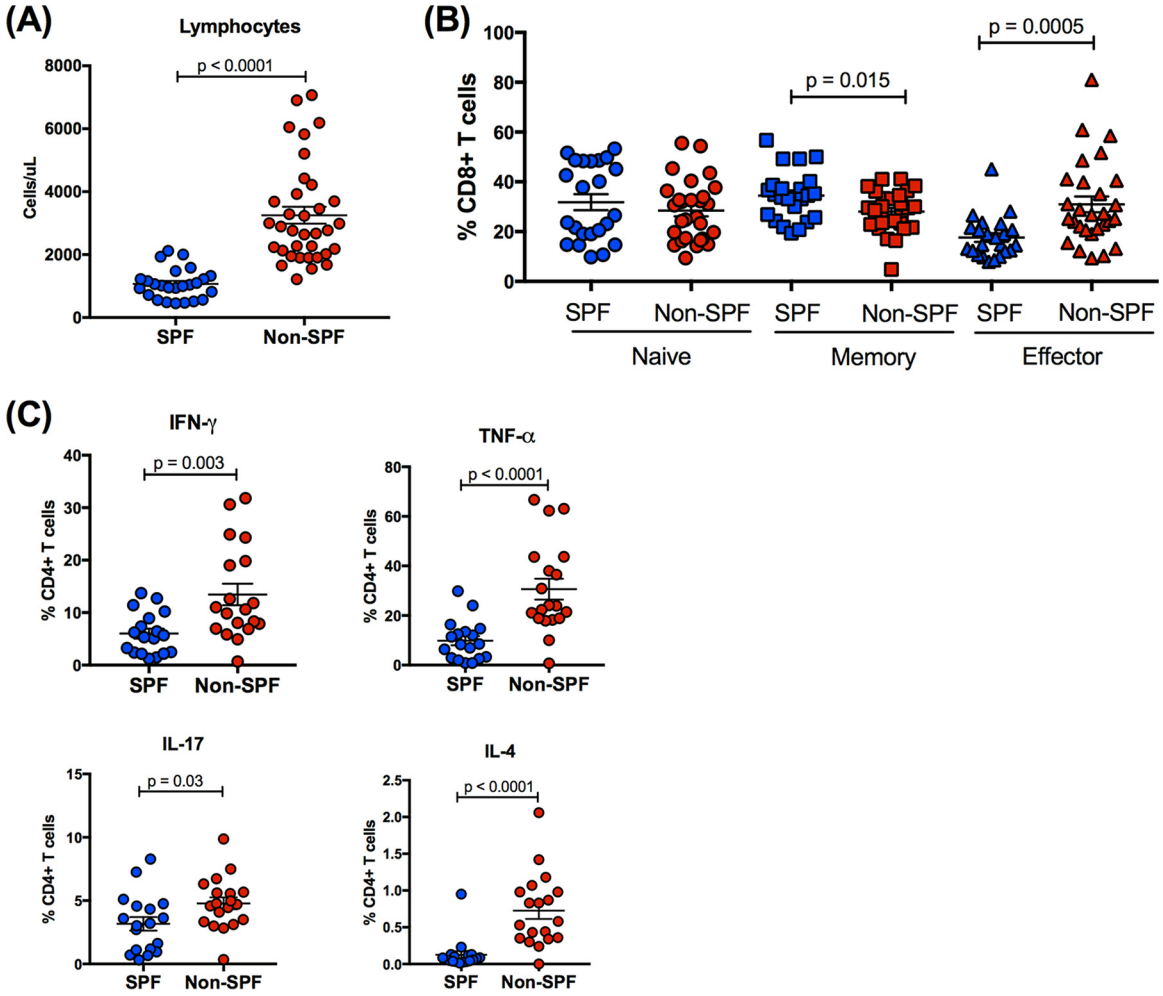


**FIG 5** Altered immune cell populations in outdoor rhesus macaques with subclinical CMV infection. (A) Complete blood counts were performed to enumerate circulating lymphocytes in SPF ($n = 25$) and non-SPF ($n = 35$) macaques. (B) $CD8^+$ T cell populations in peripheral blood were examined by flow cytometry. Naive ($CD28^+$ CD95-), memory ($CD28^+$ $CD95^+$), and effector ($CD28^-$ $CD95^+$) $CD8^+$ T cells were identified. (C) The functional potential of $CD4^+$ T helper cell subsets was measured by intracellular cytokine staining following mitogenic stimulation of PBMC from SPF ($n = 18$) and non-SPF ($n = 19$) macaques. Data are shown as means ± the SEM.

CMV-specific $CD4^+$ and $CD8^+$ T cells producing gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) in non-SPF animals (see Fig. S3 in the supplemental material). However, non-SPF animals harboring subclinical viral infections had significantly higher frequencies of Th1, Th2, and Th17 $CD4^+$ T cells as determined by intracellular cytokine immunostaining following *ex vivo* mitogenic stimulation. The proportions of IFN-γ- and TNF-α-producing cells were 2- and 3-fold higher in non-SPF compared to SPF animals. Interestingly, SPF animals had very few interleukin-4 (IL-4)-producing cells, while non-SPF animals had a 7-fold-higher frequency of these cells (Fig. 5C). No apparent differences were observed for the percentages of Treg cells (see Fig. S1C in the supplemental material). Our data on higher frequencies of effector cells, cytokine-producing T cells, and absolute numbers of circulating lymphocytes in non-SPF animals suggest that SVI are major drivers for the expansion and maturation of host immunity. The numbers and distribution of circulating lymphocytes and T cell subsets from SPF and non-SPF animals from the indoor environment were compared. Surprisingly, there was no significant difference in lymphocyte numbers between SPF and non-SPF animals that were housed indoors (Fig. 6A). In fact, SPF animals housed indoors had significantly higher numbers of circulating lymphocytes compared to SPF animals housed outdoors (compare Fig. 6A and 5A).

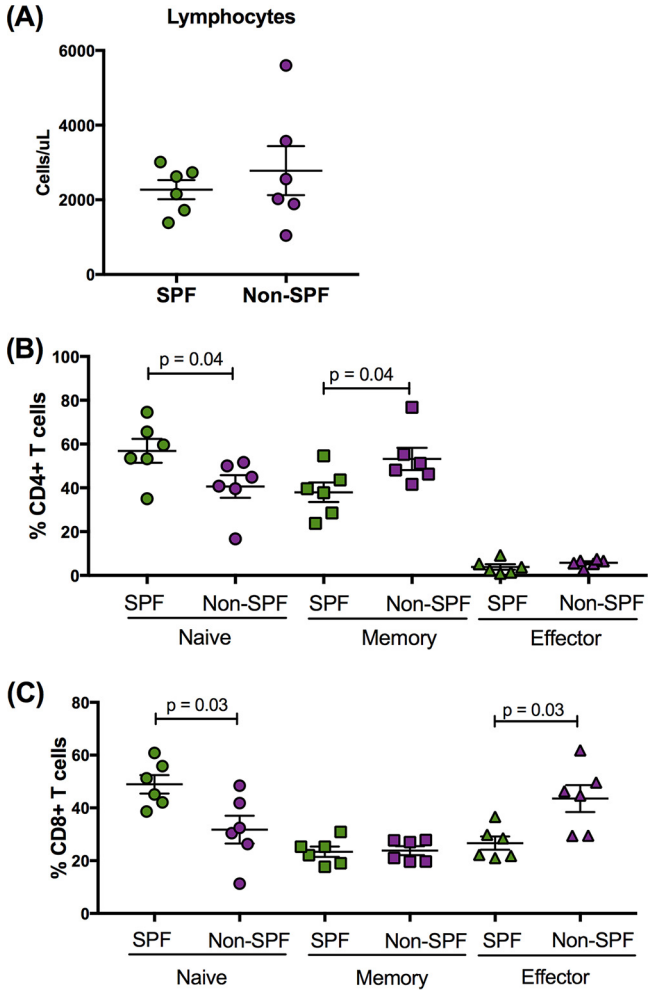


**FIG 6** Prevalence of Immune cell subsets among SPF and non-SPF animals in the indoor environment. (A) Complete blood counts were performed to enumerate circulating lymphocytes in SPF ($n = 6$) and non-SPF ($n = 6$) macaques in the indoor environment. (B and C) Naive ($CD28^+$ $CD95^-$), memory ($CD28^+$ $CD95^+$), and effector ($CD28^-$ $CD95^+$) $CD4^+$ (B) and $CD8^+$ (C) T cell populations in PBMC were examined by flow cytometry. Data are shown as group means ± the SEM.

In the indoor environment, non-SPF animals had significantly fewer naive $CD4^+$ and $CD8^+$ T cells than SPF animals (Fig. 6B and C). Non-SPF animals also exhibited an increased percentage of memory $CD4^+$ T cells (Fig. 6B) and effector $CD8^+$ T cells (Fig. 6C). No detectable differences in the $CD4^+$ and $CD8^+$ T cell proliferation were observed in indoor-housed animals (data not shown). These findings suggest that controlled hygienic indoor environment may lead to a reduced level of host immune activation.

**Marked difference in the magnitude of flu vaccine responses between SPF and non-SPF animals in the indoor environment.** To determine whether host immune responses to vaccine immunogens are different in non-SPF animals compared to SPF animals as a consequence of subclinical viral infections, SPF and non-SPF animals were immunized with an unadjuvanted trivalent influenza vaccine. Since these animals had similar immunologic and gut microbiota profiles in the indoors, they provided an excellent opportunity to examine the effect of subclinical CMV infection independent of environmental exposures. The animals received flu vaccine two times 2 weeks apart and were monitored for the subsequent 5 weeks. The SPF animals had significantly higher titers of HA antibodies than non-SPF animals (Fig. 7). Our data suggest that subclinical viral infections may influence the magnitude of primary host immune response to vaccines.

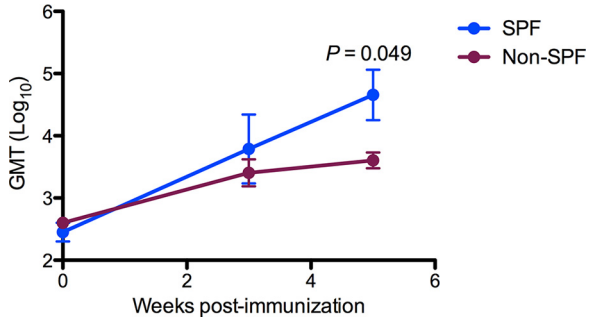


**FIG 7** Influenza H1N1 titers. The enhanced antibody responses to influenza vaccination in SPF macaques compared to non-SPF animals in the indoor environment were determined. SPF ($n = 6$) and non-SPF ($n = 6$) animals in the indoor environment were immunized with an inactivated influenza vaccine at weeks 0 and 2. Plasma samples were collected at weeks 0, 3, and 5, and H1N1-specific antibody titers were measured by ELISA. Endpoint titers are shown as log-transformed geometric means ± the SEM.

## DISCUSSION

Most of our understanding of viral interactions with the host immune system and impact of viral infections on health and disease is based on study of pathogenic viral infections that cause clinically symptomatic disease states. However, humans and animals harbor SVI that cause no detectable clinical symptoms or obvious functional abnormalities. The degree to which these silent infections may influence host immunity is just beginning to be appreciated. Chronic viral infections impact the immune system and health in humans, nonhuman primates and mice (35). A study of monozygotic human twins found that discordance for CMV infection in twin pairs resulted in significantly increased variance in more than 50% of the immune parameters measured, compared to the variance seen in CMV-negative twin pairs (2). Such findings suggest that environmental exposures, including persistent viral infections, may play a greater role in shaping host immunity than genetics.

Most immunologic studies are performed in laboratory mouse models in the indoor environment, which do not adequately capture the impact of environmental exposures and genetic variation among human populations. The inflammation profile, immune cell distribution, and gut microbiota of inbred laboratory mice are quite different from those of humans (24, 36). Nonhuman primate models are highly translational because the animals are outbred, genetically diverse, and housed outdoors, experiencing a wide range of environmental exposures. The availability of SPF and non-SPF rhesus macaques provides an excellent opportunity to investigate the impact of SVI on the host immunity and gut microbiota in the context of environmental exposures.

Our study is the first to demonstrate striking differences in the composition of the gut microbiota of nonhuman primates from the outdoor environment versus indoor environment. Human microbiome studies have highlighted the impact of the environment (e.g., geographical location) and lifestyle (e.g., diet) on gut microbiota composition (37–39). Most preclinical nonhuman primate studies have utilized indoor-housed animals, and therefore the interacting influences of environmental exposures and divergent gut microbial communities were not evaluated (40–42). We find that members of *Firmicutes* are associated with indoor housing, while *Proteobacteria* and *Bacteroidetes* members are associated with outdoor housing. Despite the expected normal variation in the gut microbiota among animals from different primate centers or animal service facilities, the proportions of *Bacteroidetes* (~58%) and *Firmicutes* (~30%) in indoor animals in our study are similar to the proportions previously reported in nonhuman primates by other investigators (40, 41). This is possibly due to the use of indoor animals in their studies. However, the proportions of these microbes in the outdoor animals (~68% for *Bacteroidetes* and only 17% for *Firmicutes*) in our study were different from the indoor animals from previously reported studies. Importantly, those compositional differences were reflected in functional changes in their microbial

metabolic potential. In general, pathways related to lipid and carbohydrate metabolism were highly enriched in the indoor animals compared to the outdoor animals. In contrast, glycan metabolism pathway was exclusively associated with outdoor animals. A higher abundance of *Firmicutes* is generally related to increased energy harvesting through the metabolism of carbohydrates (43), while a higher abundance of *Bacteroidetes* was associated with the metabolism of glycans (44).

A demonstrable difference in the microbiota composition of rhesus macaques was observed only in the context of their housing environment. We did not detect significantly different microbiota patterns according to the age in the SPF and non-SPF animals. Gut microbiota changes between young and aged have been previously reported in humans (45). However, our experimental groups did not include infants or very aged animals. Animals in our study groups were already immunologically mature and possibly developed similar microbiota profiles since they received similar diet and were housed in the same outdoor environment. The outdoor environmental exposures may include several factors, including exposure to microbes from soil and plants. It is likely that outdoor environmental exposures may play a substantial role in driving differences in microbiota profiles of animals housed in the outdoor versus indoor environment. Our data reveal new opportunities of using NHP models for preclinical studies that will be highly relevant and reflective of variations in the human population.

Previous mouse studies showed that approximately 16% of bacterial taxa differed between wild mice and inbred laboratory mice (46). Another study reported that wild mice have greater bacterial diversity than laboratory-housed mice (47). However, the wild mice are genetically highly diverse compared to laboratory inbred mice and their habitat, diet, and exposure to environmental stimuli are very different. These factors complicate the comparative analysis of the mice for differences in the gut microbiota or the presence of subclinical viral infections. Our findings emphasize the importance of identifying the baseline microbiomes of animal models associated with different environments in order to identify the clinical relevance of microbiota changes linked to health and disease.

Although dysbiosis of the gut microbiota has been reported in several diseases, there is limited information regarding the influence of asymptomatic subclinical chronic viral infections. Our study is the first to report the impact of SVI on the composition and function of the microbial community in healthy adult NHP housed outdoors. In infants or young animals, stable gut microbiota are established as their immune systems maturate and develop in response to environmental exposures and encounters with new viral and bacterial agents (48). However, animals in our study were adults with fully maturated immune system and a long-term SVI. Therefore, their gut microbiota profiles are similar and would be distinct from those from infants. Although our study predominantly consisted of female animals in the outdoor environment, they were cohoused together with other males. Therefore, we do not expect an effect of altered social patterns.

Our findings showed that differences in gut microbiota composition and functionality between SPF and non-SPF animals in the outdoor environment diminished when animals were moved to the indoor environment. The non-SPF animals showed an increased abundance of potential butyrate-producing bacteria such as *Faecalibacterium* and *Coprococcus* (members of *Clostridium* groups IV and XIVa, respectively) compared to SPF animals in the outdoor environment. This is particularly intriguing since these two microbial species are among the most abundant butyrate producers commonly found in the gastrointestinal tracts of mammals (49). It is interesting to note that severe loss of butyrate-producing bacteria is reported for several inflammatory infectious diseases, including HIV/AIDS (19) and *Clostridium difficile* infection (20), and noninfectious inflammatory diseases, including Crohn's disease and inflammatory bowel disease (21, 50). However, an increased prevalence of potential butyrate-producing microbes was detected in non-SPF animals with SVI in our study. The SPF animals at CNPRC are confirmed to be seronegative for RhCMV and simian retroviruses (SIV, simian T lymphotropic virus (STLV), SRV, and SFV), as well as B virus and rhesus rhadinovirus. In

contrast, most non-SPF animals are seropositive for SFV and RhCMV by the age of 1 year (51). Experimental infection of indoor SPF animals with CMV resulted in increased abundance of potential butyrate-producing bacteria, demonstrating that CMV enhanced host colonization by butyrate-producing commensals. However, it is possible that other SVI may also contribute to the gut microbiota changes found between SPF and non-SPF animals.

Although we did not directly measure the levels of butyrate in the gut, we found increased counts of butyryl-CoA dehydrogenase gene in the predicted metagenome of non-SPF animals, suggesting enhanced ability of butyrate production compared to SPF animals. In our study, we did not measure butyrate levels in fecal samples. In human fecal samples, acetate is the most prevalent SCFA compared to propionate and butyrate. In the gut, SCFAs are utilized by host cells, and the levels in the fecal samples do not adequately reflect SCFAs, specifically butyrate in the intestine (52).

SCFAs, including butyrate and propionate, are known to inhibit histone deacetylase. Butyrate has been shown to reactivate CMV promoter-driven expression of latent viral genes (31). Interestingly, the abundance of *Faecalibacterium* and *Lachnospiraceae* positively correlated with RhCMV-specific antibody titers in the outdoor animals. Although we did not measure CMV replication, it has been previously reported that levels of CMV-specific antibodies correlate with RhCMV shedding (34). Thus, increased colonization of butyrate-producing microbiota may correlate with recurrent CMV reactivation and viral persistence in non-SPF animals. Collectively, these findings support the hypothesis that microbiota-derived butyrate may contribute to the mechanisms of viral persistence *in vivo* by inducing low-level CMV gene expression through epigenetic modifications or activation of transcription binding sites. Concurrently, butyrate plays an important role on host physiology by inducing anti-inflammatory responses, which controls excessive viral replication (53). While butyrate is able to reactivate latent viral expression, butyrate-mediated anti-inflammatory effects may drive the balance between pro- and anti-inflammatory mechanisms during subclinical viral infections. This negative feedback to fine-tune viral replication by butyrate maintains the viral persistence *in vivo*. It is also noteworthy that the changes in the gut microbiota of animals with SVI mapped to microbes of low abundance in the gut. However, changes in these low-prevalence microbes were sufficient to impact the functional capacity of the microbiome. Future investigations are needed to elucidate how increased abundance of butyrate-producing microbes in hosts with subclinical viral infections would impact both host immunity and viral persistence.

Our data show markedly different immune cell phenotypes and functions between non-SPF animals and SPF animals in the outdoor environment. The major differences included a significant increase in the numbers of circulating lymphocytes and effector T cells. These parameters might reflect a cumulative effect of multiple antigenic exposures in the outdoor environment that are exacerbated by the presence of persistent viral infections. It is possible that SVI enhance sensitization of the animals to environmental exposures. The SPF and non-SPF animals in the indoor environment had similar levels of circulating lymphocytes, but the frequencies of naive and memory T cells were different between these two groups. It is reasonable to detect an increased percentage of memory T cells in the indoor non-SPF animals because they harbor persistent CMV infection. Although CMV infection is associated with variations in immune cell functions in humans and NHPs (9, 54), it is possible that other persistent or latent viral infections may also contribute to host immune variation. SFV is considered to be nonpathogenic to rhesus macaques (55). While intermittent reactivation and shedding of B virus in some animals has been documented (56), its impact on host immunity has not been fully characterized. All non-SPF animals in our study were seronegative for SIV and SRV (51).

Previous studies on the impact of CMV infection on host immune responses to vaccines have been inconclusive. Among elderly humans, CMV seropositivity had either no effect (10) or an adverse effect (11, 12, 14) on the antibody responses to influenza vaccination. Studies in younger human populations have also reported mixed results, including no effect due to CMV seropositivity (11), an adverse effect (12, 14), or a

**TABLE 1** Study groups

| Group | No. of subjects | Mean ± SD (range) | | Sex (no. male: no. female) |
|---|---|---|---|---|
| | | Age (yrs) | Wt (kg) | |
| Outdoor SPF | 25 | 9.3 ± 4.1 (3.25–14.0) | 9.3 ± 3.0 (4.6–19.2) | 4:21 |
| Outdoor non-SPF | 35 | 8.3 ± 4.6 (3.1–19.3) | 7.8 ± 2.7 (3.9–13.1) | 0:35 |
| Indoor SPF | 6 | 4.8 ± 1.0 (3.9–6.3) | 7.4 ± 0.6 (6.6–8.2) | 4:2 |
| Indoor non-SPF | 6 | 6.1 ± 2.6 (3.7–9.1) | 9.3 ± 3.8 (5.6–15.7) | 3:3 |

positive effect (2) on response rates to influenza vaccination. We examined host immune responses to flu vaccination in SPF and non-SPF animals housed in the indoor environment. It was remarkable that SPF and non-SPF animals in the indoor environment had very similar profiles of the gut microbiota and immune cells. The differences observed in gut microbiota composition and immune cell subset distribution between outdoor SPF and non-SPF animals diminished after their transfer to indoor housing regardless the length of time indoors, suggesting that changes are rapidly established in the indoor environment. This enabled us to examine the role of CMV and SVI on host immune responses to vaccines. We transferred animals from outdoor corrals to indoor housing (for 3 to 34 months) prior to flu vaccination. Based on H1N1-specific IgG titers, we found that non-SPF animals had lower flu-specific antibody titers than SPF animals, suggesting that the presence of subclinical viral infections modulated host immunity to vaccines.

In summary, our study demonstrates that SVI play an important role in inducing host immune variation and altering gut microbiota colonization in healthy adult rhesus macaques, both of which are accentuated in the outdoor environment. Our findings suggest the possibility that subclinical infectious viruses such as CMV sensitize hosts to environmental exposures, accentuating their impact. We also demonstrate that clinically silent viruses modulate host immune responses to vaccines. Thus, subclinical virus-driven changes contribute to host variation, at the population level, in the composition and function of the immune system.

## MATERIALS AND METHODS

**Animals and Sample collection.** Rhesus macaques (*Macaca mulatta*) were obtained from the CNPRC. This study was performed in accordance with the recommendations of the Public Health Services (PHS) Policy on Humane Care and Use of Laboratory Animals. All procedures were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the University of California, Davis. Outbred rhesus macaques were obtained from the SPF level 2 and non-SPF colonies at the CNPRC. The SPF animals are seronegative for SRV, SIV, STLV, B virus, RhCMV, and SFV. The first part of the study involved a total of 60 rhesus macaques (25 SPF and 35 non-SPF) ranging from 3 to 19 years old. Both SPF and non-SPF animals were born, reared, and housed in outdoor corrals at the CNPRC and are exposed to same diet and environment (Table 1). Animals were breast-fed during infancy. Peripheral blood samples and rectal swabs were collected, and immunophenotypic analysis and gut microbiota assessment were performed. Animals in the study did not receive any antibiotics during the 6 months prior to the sample collection.

In the second part of the study, SPF ($n = 6$) and non-SPF ($n = 6$) macaques ranging from 3 to 9 years old were immunized twice with an inactivated, unadjuvanted, influenza vaccine (Fluzone) administered via the intramuscular route with a dose of 45 $\mu$g of hemagglutinin at weeks 0 and 2. All animals were transferred from outdoor corrals to indoor housing for at least 3 to 34 months prior to the study. Peripheral blood samples were collected at day 0 (prior to the first immunization) and at weeks 3 and 5 after immunization. Serum samples were used to measure anti-hemagglutinin antibody responses at weeks 3 and 5 (3 weeks after the prime and booster immunizations), respectively.

**Flow cytometric analysis.** The distribution of T cell memory subsets in peripheral blood samples and their activation status were determined by flow cytometry as previously described (54). Tregs were characterized using the following antibodies: anti-CD3-Alexa 700 (SP34-2), anti-CD95-APC (DX2), anti-CD28-APC-H7 (CD28.2), anti-CD127-PE (HIL-7R-M21), anti-CD25–PE-Cy7 (M-A251), anti-CD8-PE-Cy5.5 (3B5), anti-CD4-BV650 (L200), and anti-CD45RA-ECD (2H4). A cell viability dye was included to discriminate live from dead cells (Invitrogen Aqua Live/Dead Fixable Dead Cell Stain). Cells were washed and permeabilized using a FOXP3 Fix/Perm kit (BioLegend), intracellularly stained with anti-Ki67-Alexa 488 (B56) and anti-FOXP3-PacBlue (206D), and fixed in phosphate-buffered saline (PBS) containing 1% paraformaldehyde. T cell phenotypes were defined by CD28 and CD95 markers: $CD28^+$ $CD95^-$ (naive), $CD28^+$ $CD95^+$ (memory), and $CD28^-$ $CD95^+$ (effector) (57).

The T cell subsets were assessed by cytokine production following mitogenic stimulation. PBMC ($10^6$ cells) were incubated for 4 h with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 $\mu$g/ml)

in complete RPMI 1640 medium and with GolgiPlug (5 $\mu$g/ml). Cells were washed and immunostained with anti-CD3-PacBlue (SP34-2), anti-CD8-PE-Cy5.5 (3B5), anti-CD4-BV650 (L200), anti-CD95-APC (DX2), and anti-CD28-APC-H7 (CD28.2). Cells were permeabilized with Cytofix/Cytoperm kit (BD Biosciences) and immunostained with anti-IL-17-PE (eBio64DEC17), anti-IFN-$\gamma$-PE-Cy7 (B27), anti-TNF-$\alpha$-Alexa Fluor 700 (Mab11), and anti-IL-4 FITC (MP4-25D2). Cells were fixed in PBS containing 1% paraformaldehyde. Data were acquired on LSR II or Fortessa cytometers (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**ELISA.** The serological assessment of RhCMV was performed in plasma samples from non-SPF animals using enzyme-linked immunosorbent assay (ELISA) with RhCMV-binding IgG antibodies as previously described (58). Data are shown in relative units, which were calculated based on an eight-point standard curve with a range of 1:100 to 1:12,800 dilutions with the 1:400-diluted assay control standard arbitrarily set to 100 Ig units. The optical density (OD) values of the test samples were converted to IgG units by a log-log regression model.

Anti-influenza A antibody levels were quantified following flu vaccination. The 96-well plates (Immulon 4 HBX; Thermo Scientific) were coated with 0.25 $\mu$g/ml of gamma radiation-inactivated A/California/7/2009 influenza virus (influenza A H1N1; Meridian Life Science). Plasma samples were serially diluted from 1:400 to 1:256,000 and incubated for 2 h at 25°C, followed by the addition of horseradish peroxidase-conjugated goat anti-monkey IgG polyclonal antibodies in 1% bovine serum albumin in PBS plus Tween. Wells were reacted with TMB substrate (Sigma) for 20 min and stopped with 1 N sulfuric acid. The absorbance was read at an OD of 450 nm ($OD_{450}$) and $OD_{595}$, and the $OD_{450}$ was subtracted from the background $OD_{595}$. Endpoint antibody titers are defined as the dilution with an OD value greater than the preimmunization sample plus 2 standard deviations.

**Gut microbiota analysis and 16S rRNA sequencing.** The gut microbiota was assessed in total DNA from rectal swabs (MoBio Power Soil kit). Amplification of the V4 region of the 16S rRNA gene was performed using DNA template and primers 515F and 806R as previously described (23). A unique combination of these primers was used to barcode each sample. PCR mixtures contained 1 U of Kapa2G Robust Hot Start polymerase (Kapa Biosystems), 10 pmol of each primer, and 1 $\mu$l (ranging from 10 to 40 $\mu$g/ml) of template DNA. The final PCR products were purified using Axyprep magnetic beads (Axygen) and quantified on the Qubit instrument using a Qubit high-sensitivity DNA kit (Invitrogen), and individual amplicons were pooled in equal concentrations. The pooled library was purified using a 1.5% Pippin prep gel (Sage Science), the quality was assessed using the Agilent Bioanalyzer 2100 High Sensitivity DNA assay, and 250-bp paired-end sequencing was performed an Illumina MiSeq instrument. Sequencing reads were trimmed of their barcodes, demultiplexed, and combined using custom Perl scripts. Chimeras sequences were removed using VSEARCH (59) and the resulting reads were aligned to the Greengenes (release 13_8) database using Qiime (60) with the default parameters. The resulting Biom tables were rarefied at 4,000 reads to avoid biases due to different sequencing depths, filtered to remove low-abundance operational taxonomic units (relative abundance $<$ 0.1% in all samples), and used for downstream analysis.

**RhCMV experimental infection.** The SPF rhesus macaques ($n$ = 4) were subcutaneously infected with $10^5$ PFU of RhCMV (UCD52 strain) as previously described (61). Peripheral blood samples and rectal swabs were collected at 2, 3, 4, and 16 weeks postinfection, as well as preinfection. Plasma samples were evaluated for the levels of RhCMV-specific antibody titers by ELISA as previously described (62, 63). The composition of gut microbiota was determined as described above.

**Statistical analysis.** Unpaired $t$ tests or Mann-Whitney tests were used for comparisons of T cell phenotypes, RhCMV antibody response, and influenza antibody responses (GraphPad Prism). Differences in microbial abundance between SPF and non-SPF animals were analyzed by using a two-tailed Mann-Whitney test with FDR correction. Differences between groups were considered significant when the $P$ value was $\leq$0.05 and the FDR-corrected $P$ value (q-value) was $\leq$0.1. Microbiome differences between indoor and outdoor were analyzed by using LDA effect size (LEfSe). Spearman correlations and correlation heatmaps were performed with the R statistical software. Correlations were considered significant when the FDR-adjusted $P$ value was $<$0.05. Unweighted and weighted UniFrac PCoA was performed with R package phyloseq (64), and PERMANOVA was performed to determine statistically significant differences between groups. Friedman test (nonparametric ANOVA with repeated measures), followed by Dunn's posttest was used for comparisons of microbiota changes over the course of infection time. Functional analysis of microbial communities was predicted by using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (27) and LEfSe analysis (threshold of 1.5) were performed to identify the pathways that were statistically different between the groups.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.00167-18.

**SUPPLEMENTAL FILE 1,** PDF file, 0.4 MB.

## ACKNOWLEDGMENTS

We thank Camille Scott, Nicole Narayan, Yara Paiva, and Matthew Rolston for support of microbiome studies. We thank Ian McHardy for comments on the manuscript. We thank the CNPRC staff, Linda Hirst and Wilhelm von Morgenland, for their support of the NHP studies.

This study was supported by National Institutes of Health grants AI120739, AI123105, and OD P51 OD011107. C.S.R. received a postdoctoral fellowship from the CNPq, Brazil. We declare that we have no competing interests.

## REFERENCES


1. Brodin P, Davis MM. 2017. Human immune system variation. Nat Rev Immunol 17:21–29. https://doi.org/10.1038/nri.2016.125.
2. Brodin P, Jojic V, Gao T, Bhattacharya S, Angel CJ, Furman D, Shen-Orr S, Dekker CL, Swan GE, Butte AJ, Maecker HT, Davis MM. 2015. Variation in the human immune system is largely driven by non-heritable influences. Cell 160:37–47. https://doi.org/10.1016/j.cell.2014.12.020.
3. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. 2011. Human nutrition, the gut microbiome and the immune system. Nature 474:327–336. https://doi.org/10.1038/nature10213.
4. Liston A, Carr EJ, Linterman MA. 2016. Shaping variation in the human immune system. Trends Immunol 37:637–646. https://doi.org/10.1016/j.it.2016.08.002.
5. Cannon MJ, Schmid DS, Hyde TB. 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev Med Virol 20:202–213. https://doi.org/10.1002/rmv.655.
6. Boeckh M, Geballe AP. 2011. Cytomegalovirus: pathogen, paradigm, and puzzle. J Clin Invest 121:1673–1680. https://doi.org/10.1172/JCI45449.
7. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, Sleath PR, Grabstein KH, Hosken NA, Kern F, Nelson JA, Picker LJ. 2005. Broadly targeted human cytomegalovirus-specific $CD4^+$ and $CD8^+$ T cells dominate the memory compartments of exposed subjects. J Exp Med 202:673–685. https://doi.org/10.1084/jem.20050882.
8. Vescovini R, Biasini C, Fagnoni FF, Telera AR, Zanlari L, Pedrazzoni M, Bucci L, Monti D, Medici MC, Chezzi C, Franceschi C, Sansoni P. 2007. Massive load of functional effector $CD4^+$ and $CD8^+$ T cells against cytomegalovirus in very old subjects. J Immunol 179:4283–4291. https://doi.org/10.4049/jimmunol.179.6.4283.
9. Chidrawar S, Khan N, Wei W, McLarnon A, Smith N, Nayak L, Moss P. 2009. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. Clin Exp Immunol 155:423–432. https://doi.org/10.1111/j.1365-2249.2008.03785.x.
10. den Elzen WP, Vossen AC, Cools HJ, Westendorp RG, Kroes AC, Gussekloo J. 2011. Cytomegalovirus infection and responsiveness to influenza vaccination in elderly residents of long-term care facilities. Vaccine 29:4869–4874. https://doi.org/10.1016/j.vaccine.2011.03.086.
11. Derhovanessian E, Theeten H, Hahnel K, Van Damme P, Cools N, Pawelec G. 2013. Cytomegalovirus-associated accumulation of late-differentiated CD4 T cells correlates with poor humoral response to influenza vaccination. Vaccine 31:685–690. https://doi.org/10.1016/j.vaccine.2012.11.041.
12. Frasca D, Diaz A, Romero M, Landin AM, Blomberg BB. 2015. Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine. Vaccine 33:1433–1439. https://doi.org/10.1016/j.vaccine.2015.01.071.
13. Furman D, Jojic V, Sharma S, Shen-Orr SS, Angel CJ, Onengut-Gumuscu S, Kidd BA, Maecker HT, Concannon P, Dekker CL, Thomas PG, Davis MM. 2015. Cytomegalovirus infection enhances the immune response to influenza. Sci Transl Med 7:281ra243. https://doi.org/10.1126/scitranslmed.aaa2293.
14. Trzonkowski P, Mysliwska J, Szmit E, Wieckiewicz J, Lukaszuk K, Brydak LB, Machala M, Mysliwski A. 2003. Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination: an impact of immunosenescence. Vaccine 21:3826–3836. https://doi.org/10.1016/S0264-410X(03)00309-8.
15. Rooks MG, Garrett WS. 2016. Gut microbiota, metabolites and host immunity. Nat Rev Immunol 16:341–352. https://doi.org/10.1038/nri.2016.42.
16. Oh JZ, Ravindran R, Chassaing B, Carvalho FA, Maddur MS, Bower M, Hakimpour P, Gill KP, Nakaya HI, Yarovinsky F, Sartor RB, Gewirtz AT, Pulendran B. 2014. TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. Immunity 41:478–492. https://doi.org/10.1016/j.immuni.2014.08.009.
17. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, Paley MA, Antenus M, Williams KL, Erikson J, Wherry EJ, Artis D. 2012. Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity 37:158–170. https://doi.org/10.1016/j.immuni.2012.04.011.
18. Tanaka K, Sawamura S, Satoh T, Kobayashi K, Noda S. 2007. Role of the indigenous microbiota in maintaining the virus-specific CD8 memory T cells in the lung of mice infected with murine cytomegalovirus. J Immunol 178:5209–5216. https://doi.org/10.4049/jimmunol.178.8.5209.
19. Dillon SM, Lee EJ, Kotter CV, Austin GL, Dong Z, Hecht DK, Gianella S, Siewe B, Smith DM, Landay AL, Robertson CE, Frank DN, Wilson CC. 2014. An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. Mucosal Immunol 7:983–994. https://doi.org/10.1038/mi.2013.116.
20. Antharam VC, Li EC, Ishmael A, Sharma A, Mai V, Rand KH, Wang GP. 2013. Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhea. J Clin Microbiol 51:2884–2892. https://doi.org/10.1128/JCM.00845-13.
21. Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van Treuren W, Ren B, Schwager E, Knights D, Song SJ, Yassour M, Morgan XC, Kostic AD, Luo C, Gonzalez A, McDonald D, Haberman Y, Walters T, Baker S, Rosh J, Stephens M, Heyman M, Markowitz J, Baldassano R, Griffiths A, Sylvester F, Mack D, Kim S, Crandall W, Hyams J, Huttenhower C, Knight R, Xavier RJ. 2014. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe 15:382–392. https://doi.org/10.1016/j.chom.2014.02.005.
22. Zevin AS, McKinnon L, Burgener A, Klatt NR. 2016. Microbial translocation and microbiome dysbiosis in HIV-associated immune activation. Curr Opin HIV AIDS 11:182–190. https://doi.org/10.1097/COH.0000000000000234.
23. Glavan TW, Gaulke CA, Santos Rocha C, Sankaran-Walters S, Hirao LA, Raffatellu M, Jiang G, Baumler AJ, Goulart LR, Dandekar S. 2016. Gut immune dysfunction through impaired innate pattern recognition receptor expression and gut microbiota dysbiosis in chronic SIV infection. Mucosal Immunol 9:677–688. https://doi.org/10.1038/mi.2015.92.
24. Mestas J, Hughes CC. 2004. Of mice and not men: differences between mouse and human immunology. J Immunol 172:2731–2738. https://doi.org/10.4049/jimmunol.172.5.2731.
25. Tao L, Reese TA. 2017. Making mouse models that reflect human immune responses. Trends Immunol 38:181–193. https://doi.org/10.1016/j.it.2016.12.007.
26. Phillips KA, Bales KL, Capitanio JP, Conley A, Czoty PW, t Hart BA, Hopkins WD, Hu SL, Miller LA, Nader MA, Nathanielsz PW, Rogers J, Shively CA, Voytko ML. 2014. Why primate models matter. Am J Primatol 76:801–827. https://doi.org/10.1002/ajp.22281.
27. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31:814–821. https://doi.org/10.1038/nbt.2676.
28. Watanabe Y, Nagai F, Morotomi M. 2012. Characterization of *Phascolarctobacterium succinatutens* sp. nov., an asaccharolytic, succinate-utilizing bacterium isolated from human feces. Appl Environ Microbiol 78:511–518. https://doi.org/10.1128/AEM.06035-11.
29. Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ. 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 217:133–139. https://doi.org/10.1111/j.1574-6968.2002.tb11467.x.
30. Vital M, Howe AC, Tiedje JM. 2014. Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. mBio 5:e00889. https://doi.org/10.1128/mBio.00889-14.
31. Choi KH, Basma H, Singh J, Cheng PW. 2005. Activation of CMV promoter-controlled glycosyltransferase and beta-galactosidase glycogenes by butyrate, tricostatin A, and 5-aza-2′-deoxycytidine. Glycoconj J 22:63–69. https://doi.org/10.1007/s10719-005-0326-1.
32. Gorres KL, Daigle D, Mohanram S, Miller G. 2014. Activation and repression of Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus lytic cycles by short- and medium-chain fatty acids. J Virol 88:8028–8044. https://doi.org/10.1128/JVI.00722-14.

33. Imai K, Yamada K, Tamura M, Ochiai K, Okamoto T. 2012. Reactivation of latent HIV-1 by a wide variety of butyric acid-producing bacteria. Cell Mol Life Sci 69:2583–2592. https://doi.org/10.1007/s00018-012-0936-2.
34. Oxford KL, Dela Pena-Ponce MGA, Jensen K, Eberhardt MK, Spinner A, Van Rompay KK, Rigdon J, Mollan KR, Krishnan VV, Hudgens MG, Barry PA, De Paris K. 2015. The interplay between immune maturation, age, chronic viral infection and environment. Immun Ageing 12:3. https://doi.org/10.1186/s12979-015-0030-3.
35. Virgin HW, Wherry EJ, Ahmed R. 2009. Redefining chronic viral infection. Cell 138:30–50. https://doi.org/10.1016/j.cell.2009.06.036.
36. Nguyen TL, Vieira-Silva S, Liston A, Raes J. 2015. How informative is the mouse for human gut microbiota research? Dis Model Mech 8:1–16. https://doi.org/10.1242/dmm.017400.
37. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. 2012. Diversity, stability, and resilience of the human gut microbiota. Nature 489:220–230. https://doi.org/10.1038/nature11550.
38. Conlon MA, Bird AR. 2014. The impact of diet and lifestyle on gut microbiota and human health. Nutrients 7:17–44. https://doi.org/10.3390/nu7010017.
39. Bhute S, Pande P, Shetty SA, Shelar R, Mane S, Kumbhare SV, Gawali A, Makhani H, Navandar M, Dhotre D, Lubree H, Agarwal D, Patil R, Ozarkar S, Ghaskadbi S, Yajnik C, Juvekar S, Makharia GK, Shouche YS. 2016. Molecular characterization and meta-analysis of gut microbial communities illustrate enrichment of *Prevotella* and *Megasphaera* in Indian subjects. Front Microbiol 7:660. https://doi.org/10.3389/fmicb.2016.00660.
40. McKenna P, Hoffmann C, Minkah N, Aye PP, Lackner A, Liu Z, Lozupone CA, Hamady M, Knight R, Bushman FD. 2008. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. PLoS Pathog 4:e20. https://doi.org/10.1371/journal.ppat.0040020.
41. Klase Z, Ortiz A, Deleage C, Mudd JC, Quinones M, Schwartzman E, Klatt NR, Canary L, Estes JD, Brenchley JM. 2015. Dysbiotic bacteria translocate in progressive SIV infection. Mucosal Immunol 8:1009–1020. https://doi.org/10.1038/mi.2014.128.
42. Yasuda K, Oh K, Ren B, Tickle TL, Franzosa EA, Wachtman LM, Miller AD, Westmoreland SV, Mansfield KG, Vallender EJ, Miller GM, Rowlett JK, Gevers D, Huttenhower C, Morgan XC. 2015. Biogeography of the intestinal mucosal and lumenal microbiome in the rhesus macaque. Cell Host Microbe 17:385–391. https://doi.org/10.1016/j.chom.2015.01.015.
43. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1031. https://doi.org/10.1038/nature05414.
44. Martens EC, Koropatkin NM, Smith TJ, Gordon JI. 2009. Complex glycan catabolism by the human gut microbiota: the *Bacteroidetes* Sus-like paradigm. J Biol Chem 284:24673–24677. https://doi.org/10.1074/jbc.R109.022848.
45. Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ, Abe F, Osawa R. 2016. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol 16:90. https://doi.org/10.1186/s12866-016-0708-5.
46. Kreisinger J, Cizkova D, Vohanka J, Pialek J. 2014. Gastrointestinal microbiota of wild and inbred individuals of two house mouse subspecies assessed using high-throughput parallel pyrosequencing. Mol Ecol 23:5048–5060. https://doi.org/10.1111/mec.12909.
47. Linnenbrink M, Wang J, Hardouin EA, Kunzel S, Metzler D, Baines JF. 2013. The role of biogeography in shaping diversity of the intestinal microbiota in house mice. Mol Ecol 22:1904–1916. https://doi.org/10.1111/mec.12206.
48. Rodriguez JM, Murphy K, Stanton C, Ross RP, Kober OI, Juge N, Avershina E, Rudi K, Narbad A, Jenmalm MC, Marchesi JR, Collado MC. 2015. The composition of the gut microbiota throughout life, with an emphasis on early life. Microb Ecol Health Dis 26:26050. https://doi.org/10.3402/mehd.v26.26050.
49. Louis P, Flint HJ. 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett 294:1–8. https://doi.org/10.1111/j.1574-6968.2009.01514.x.
50. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottiere HM, Dore J, Marteau P, Seksik P, Langella P. 2008. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn's disease patients. Proc Natl Acad Sci U S A 105:16731–16736. https://doi.org/10.1073/pnas.0804812105.
51. Yee JL, Vanderford TH, Didier ES, Gray S, Lewis A, Roberts J, Taylor K, Bohm RP. 2016. Specific pathogen free macaque colonies: a review of principles and recent advances for viral testing and colony management. J Med Primatol 45:55–78. https://doi.org/10.1111/jmp.12209.
52. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 54:2325–2340. https://doi.org/10.1194/jlr.R036012.
53. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504:451–455. https://doi.org/10.1038/nature12726.
54. Oxford KL, Dela Pena-Ponce MG, Jensen K, Eberhardt MK, Spinner A, Van Rompay KK, Rigdon J, Mollan KR, Krishnan VV, Hudgens MG, Barry PA, De Paris K. 2015. The interplay between immune maturation, age, chronic viral infection and environment. Immun Ageing 12:3. https://doi.org/10.1186/s12979-015-0030-3.
55. Murray SM, Picker LJ, Axthelm MK, Hudkins K, Alpers CE, Linial ML. 2008. Replication in a superficial epithelial cell niche explains the lack of pathogenicity of primate foamy virus infections. J Virol 82:5981–5985. https://doi.org/10.1128/JVI.00367-08.
56. Hilliard J. 2007. Chapter 57. Monkey B virus. *In* Arvin A, Campadella-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, Yamanishi K (ed), Human herpesviruses: biology, therapy, and immunoprophylaxis. Cambridge University Press, Cambridge, United Kingdom. https://www.ncbi.nlm.nih.gov/books/NBK47426.
57. Pitcher CJ, Hagen SI, Walker JM, Lum R, Mitchell BL, Maino VC, Axthelm MK, Picker LJ. 2002. Development and homeostasis of T cell memory in rhesus macaque. J Immunol 168:29–43. https://doi.org/10.4049/jimmunol.168.1.29.
58. Chang WL, Barry PA. 2010. Attenuation of innate immunity by cytomegalovirus IL-10 establishes a long-term deficit of adaptive antiviral immunity. Proc Natl Acad Sci U S A 107:22647–22652. https://doi.org/10.1073/pnas.1013794108.
59. Rognes T, Flouri T, Nichols B, Quince C, Mahe F. 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4:e2584. https://doi.org/10.7717/peerj.2584.
60. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303.
61. Abel K, Martinez J, Yue Y, Lacey SF, Wang Z, Strelow L, Dasgupta A, Li Z, Schmidt KA, Oxford KL, Assaf B, Longmate JA, Diamond DJ, Barry PA. 2011. Vaccine-induced control of viral shedding following rhesus cytomegalovirus challenge in rhesus macaques. J Virol 85:2878–2890. https://doi.org/10.1128/JVI.00883-10.
62. Lockridge KM, Sequar G, Zhou SS, Yue Y, Mandell CP, Barry PA. 1999. Pathogenesis of experimental rhesus cytomegalovirus infection. J Virol 73:9576–9583.
63. Eberhardt MK, Deshpande A, Chang WL, Barthold SW, Walter MR, Barry PA. 2013. Vaccination against a virus-encoded cytokine significantly restricts viral challenge. J Virol 87:11323–11331. https://doi.org/10.1128/JVI.01925-13.
64. McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8:e61217. https://doi.org/10.1371/journal.pone.0061217.