Rhythmicity of intestinal IgA responses confers oscillatory commensal microbiota mutualism

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Interactions between the mammalian host and commensal microbiota are enforced through a range of immune responses that confer metabolic benefits and promote tissue health and homeostasis. Immunoglobulin A (IgA) responses directly determine the composition of commensal species that colonize the intestinal tract but require substantial metabolic resources to fuel antibody production by tissue-resident plasma cells. Here, we demonstrate that IgA responses are subject to diurnal regulation over the course of a circadian day. Specifically, the magnitude of IgA secretion, as well as the transcriptome of intestinal IgA+ plasma cells, was found to exhibit rhythmicity. Oscillatory IgA responses were found to be entrained by time of feeding and were also found to be in part coordinated by the plasma cell–intrinsic circadian clock via deletion of the master clock gene Arntl. Moreover, reciprocal interactions between the host and microbiota dictated oscillatory dynamics among the commensal microbial community and its associated transcriptional and metabolic activity in an IgA-dependent manner. Together, our findings suggest that circadian networks comprising intestinal IgA, diet, and the microbiota converge to align circadian biology in the intestinal tract and to ensure host-microbial mutualism.

INTRODUCTION

Multiple mammalian species have evolved to maintain a finely balanced relationship with tissue-resident commensal bacteria that is mutually beneficial and critical for tissue homeostasis and the health of the organism. The commensal microbiota confers a multitude of mutalistic functions to mammalian hosts via the provision of complementary metabolic activity, regulation of immune cell maturation and function, and colonization resistance that prevents outgrowth of pathogenic microbes (1–4). Healthy interactions between the host and commensal microbes are dynamically regulated and determined via complex cross-talk between the microbiota, the intestinal immune system, and nutritional cues derived from the diet. Conversely, disruption of this network through changes in lifestyle, diet, infection, or antibiotic use can precipitate the onset or progression of metabolic and inflammatory diseases (2, 4–6).

Immunoglobulin A (IgA) is a specialized antibody isotype that acts to regulate commensal bacteria community composition, tissue and niche residence, and microbial gene expression (7–10). Within mucosal barrier tissues, IgA is the dominant antibody isotype and is produced in a dimeric form bound by a J-chain linker that facilitates its selective transport across the intact intestinal epithelium and secretion into the intestinal lumen (7–9). IgA is produced by tissue-resident plasma cells (IgA+ PCs) predominantly found within the small intestine (11) and at higher quantities than any other antibody isotype at homeostasis—with estimates suggesting that several grams of IgA are produced per day in healthy humans (12). PCs are terminally differentiated antibody-secreting lymphocytes of the B cell lineage that dedicate the vast majority of their cellular capacity to expanded organelle function required to power antibody translation and secretion (13, 14). In line with this, the differentiation of a class-switched B cell to PC is associated with a marked increase in cell-intrinsic cellular metabolism and nutrient transport (14, 15). Moreover, emerging evidence suggests that changes in nutrition and diet can potently perturb IgA responses in the intestinal tract, with consequences for the microbiota and whole-body metabolism (9, 16, 17).

Together, these findings highlight the considerable metabolic requirements of maintaining mucosal antibody responses to reinforce homeostatic host-commensal bacteria interactions and mutualism. To minimize the energetic cost of such metabolically demanding biology, many species have evolved dynamic regulatory mechanisms including the regulation of physiological processes through circadian rhythms. Circadian rhythmicity acts to align biological function with diurnal light cycles and feeding activity, thus temporally regulating and aligning demanding systems with active periods—associated with feeding activity and potential immune challenges—or periods of rest. Mechanistically, this is controlled by a hierarchically layered series of circadian clocks, including the light-sensing suprachiasmatic nucleus of the brain and cell-intrinsic clocks present across a broad range of cell types in peripheral organs, which can be entrained by hormonal, neuronal,
dietary cues (18–20). At the molecular level, this is controlled by a transcriptional feedback loop mediated by a series of core clock genes that counterregulate their own transcription—thus imprinting rhythmicity—while also modulating a wider signature of genes to alter cell function (19). It is now appreciated that many immune cells exhibit cell-intrinsic circadian-mediated control of cell migration and magnitude of effector functions (21). Furthermore, circadian misalignment—through altered dietary composition and feeding times, jet lag, or shift work—has been associated with a number of metabolic and inflammatory diseases, suggesting that a better understanding of circadian regulation of immunity will have therapeutic implications. However, the role of circadian rhythms in modulating intestinal immune cross-talk with the microbiota has only recently begun to be explored (22) and yet remains incompletely understood.

Recent advances have also revealed diurnal oscillatory changes in the composition and activity of the commensal microbiota itself, in part imprinted via immune pressures (23–27). It has also been proposed that bacteria may have analogous circadian clock machinery (28), suggesting that circadian rhythmicity and oscillatory biology may have evolved across species to bidirectionally regulate microbial mutualism with the mammalian host. Here, we report diurnal rhythmicity of the secretory IgA response and the IgA⁺ PC transcriptome and demonstrate roles for both the cell-intrinsic circadian clock machinery and cell-extrinsic feeding cues in aligning IgA responses. Critically, bidirectional interactions between the host immune system and microbiota act to entrain rhythmicity in IgA and regulate oscillations in the composition and metabolic activity of the commensal microbiota, thus highlighting circadian regulation of the immune system and microbiota as a key determinant of microbial mutualism.

RESULTS
Intestinal IgA responses exhibit diurnal rhythmicity
We hypothesized that energetically demanding intestinal IgA responses may be subject to diurnal regulation. To test this, we assessed the levels of secretory IgA within the feces of a single cohort of C57BL/6 mice at five time points over a 24-hour day (Zeitgeber times; ZT0, ZT6, ZT12, and ZT18). The concentration of IgA detected in the feces was found to exhibit significant and marked variation over the day (P < 0.0001 by JTK_CYCLE analysis; Fig. 1A), suggestive of diurnal oscillatory activity, which remained evident after normalizing for minor variations in total protein content between samples (Fig. S1A). In contrast, we did not observe time-of-day differences in the frequency or cell numbers of tissue-resident IgA⁺ PCs within the small intestine or colonic lamina propria (Fig. 1, B and C, and fig. S1, B to D), nor were diurnal oscillations observed in Peyer’s patch–associated IgA class-switched germinal center (GC) B cells (fig. S1, E to G). Because IgA⁺ PC numbers and intestinal IgA secretion are most enriched in the small intestine (11), we next determined the intrinsic capacity of IgA⁺ PCs sorted at different times of the day to secrete IgA ex vivo. As expected, IgA⁺ PCs secreted high amounts of IgA into culture supernatants, unlike equal numbers of sort-purified IgD⁺ B cells or IgA⁺ B cells (fig. S1H). IgA⁺ PCs from ZT0 secreted significantly higher IgA than equal numbers of cells sort-purified at ZT12 (Fig. 1D), suggesting that the capacity of IgA⁺ PCs to secrete IgA—as opposed to the numbers of IgA⁺ PCs in the intestine—may determine diurnal rhythms in IgA secretion, as observed in the feces (Fig. 1A).

To further investigate the nature of diurnal regulation of IgA⁺ PC responses, we sort-purified small intestinal IgA⁺ PCs at ZT0, ZT6, ZT12, and ZT18 and performed bulk RNA sequencing (RNA-seq); of the ~16,000 transcripts detected within our samples, 2713 genes were found to exhibit highly significant time-of-day differences and oscillatory patterns after adjusting for false discovery rate (JTK_CYCLE analysis, Benjamini-Hochberg q value (BHQ) < 0.01), equivalent to ~16% of the observed transcriptome (Fig. 1E and fig. S2A—top 50 differentially expressed genes and data file S1). Gene Ontology (GO) term enrichment of highly oscillatory genes revealed pathways involved in Cell Cycle, Protein Translation, Metabolism, and Rhythmic Process (Fig. 1F). Oscillations were detected in the expression of key genes involved in IgA⁺ PC phenotype and transcriptional regulation (Fig. 1G), suggesting the presence of external activating signals and cell-cell cross-talk pathways known to influence antibody secretory activity (Fig. 1H), and metabolic activity and cholesterol biosynthesis pathways (Fig. 1I and fig. S2, B to G). In line with this, we also observed time-of-day differences in the expression of the PC-associated proteins Xbp1s and CD138 (fig. S2, H to L). Together, these findings suggest that IgA secretion and IgA⁺ PC–intrinsic transcriptional activity within the intestinal tract exhibit diurnal rhythmicity—and provoked the possibility of potential circadian entrainment.

Cell-intrinsic circadian clock function is required for PC transcriptional rhythmicity, but not rhythmic IgA secretion
Diurnal regulation of oscillatory transcriptional activity and function in both nonimmune and immune cells is controlled in part by the cell-intrinsic “clock”—a transcriptional–translational feedback loop mediated by core clock proteins, including CLOCK, Bmal1 (encoded by Arntl), Rev-erba (Nr1d1), Period (Per1/2), and Cryptochrome (Cry1/2). IgA⁺ PCs were found to have significant oscillations in the expression of Arntl, Nr1d1, and Per2 by RNA-seq (fig. S3A), which was independently validated via reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2A). As expected, expression of Arntl within IgA⁺ PCs was found to oscillate in antiphase to Nr1d1 and Per2 over the circadian day, mirroring expression patterns in control liver tissue (fig. S3B). In contrast, sort-purified naïve IgD⁺ B cells displayed no evidence of rhythmic expression of Arntl or Per2, although they unexpectedly exhibited comparable oscillatory expression of Nr1d1 (fig. S3C). To determine the role of this cell-intrinsic circadian clock in regulating IgA secretion within the intestine, we generated conditional knockout mice in which Bmal1 was deleted within the B cell and PC lineage (Mb1Cre × ArntlB/B). Efficient deletion of Arntl and disruption of associated clock gene transcription were confirmed in IgA⁺ PCs by RT-PCR (fig. S3, D and E), whereas IgA⁺ PC frequencies and numbers were found to be unaffected by disruption of the cell-intrinsic circadian clock (Fig. 2, B and C).

To determine the role of IgA⁺ PC–intrinsic clock gene expression, we performed bulk RNA-seq on sort-purified small intestinal IgA⁺ PCs at ZT0 and ZT12 from Mb1Cre × ArntlB/B and wild-type littermate controls (Fig. 2D) and further confirmed severe disruption of time-of-day expression of the wider circadian clock gene family after deletion of Arntl (Fig. 2E). Analysis of differentially expressed genes revealed significant time-of-day–dependent signatures in control animals that were either lost (clusters I and IV),
Fig. 1. Mucosal antibody secretion and small intestinal IgA+ PC activity exhibit diurnal rhythmicity. (A) Serial fecal sampling of C57BL/6 mice at five 6-hour intervals over a circadian day (ZT0, ZT6, ZT12, ZT18, and ZT0); n = 10 (pooled from two independent datasets). Data representative of at least four independent experiments. (B) Exemplar flow plots of small intestinal CD138+ IgA+ PC, pregated as live CD45+CD3−CD5−NK1.1−MHCII+/−B220−IgD−, at ZT0 and ZT12 and (C) quantification of IgA+ PC frequencies at ZT0, ZT6, ZT12, and ZT18. (B and C) n = 5 and representative of three independent experiments. (D) Ex vivo secretion of IgA by sort-purified IgA+ PC (from ZT0 and ZT12) cultured for 18 hours. Data pooled from two independent experiments; n = 8. (E) Heatmap of significantly oscillatory genes (JTK_CYCLE, P < 0.01) identified from bulk RNA-seq of sort-purified small intestinal IgA+ PC taken at ZT0, ZT6, ZT12, and ZT18; z score of average relative gene expression [fragments per kilobase of transcript per million mapped reads (FPKM)] values of n = 5 per time point. (F) GO term pathway enrichment analysis on oscillatory gene signatures. Selected relative expression (FPKM) values for oscillatory gene signatures related to (G) PC function, survival, and identity, (H) extrinsic survival and antibody secretion signals, and (I) cellular metabolism; values representative of n = 5 per time point. P values were determined using JTK_CYCLE, with the exception of (D), which was determined via a parametric, unpaired t test. All data shown as ±SEM; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
Fig. 2. Rhythmic IgA⁺ PC activity is in part dictated by the cell-intrinsic circadian clock. (A) Relative expression of circadian clock genes in sort-purified small intestinal IgA⁺ PC at ZT0, ZT6, ZT12, and ZT18 (ZT0 double-plotted), determined by RT-PCR. n = 10 (pooled from two independent experimental cohorts). Data representative of at least three independent experiments. (B) Frequency and (C) numbers of small intestinal IgA⁺ PC in Mb1Cre/+ × Arntlfl/fl mice in comparison with Mb1+/+ × Arntlfl/fl littermate control animals; n = 5, representative of two independent experiments. NS, not significant. (D) Heatmap comparison of significantly differentially expressed genes (Benjamini-Hochberg adjusted P < 0.05) identified by bulk RNA-seq of sort-purified small intestinal IgA⁺ PC at ZT0 and ZT12 and found to significantly differ between ZT0 and ZT12 in control animals. P values for (A) and (I) to (K) were determined using JTK_CYCLE, for (D) to (H) with Benjamini-Hochberg test (DESeq2, see also Materials and Methods), and for (B) and (C) with a parametric, unpaired t test. All data shown as ±SEM unless otherwise indicated; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
suppressed (clusters II and V), or retained (cluster III) in the absence of a functional intrinsic clock (Fig. 2D and data file S2). In addition, we observed a time-of-day gene signature that was significantly enhanced in conditional knockout cells when compared with controls (cluster VI). Among these signatures, we detected a loss of time-of-day differences in classical IgA+ PC–associated genes (Fig. 2F), as also identified in bulk RNA-seq analyses of wild-type IgA+ PC over four time points (Fig. 1). In contrast, whereas a proportion of metabolism-associated genes displayed a clear loss of time-of-day differences in the absence of Arntl (Fig. 2G), others—including those involved in glycolysis, amino acid transport, the malonate pathway, and cholesterol biosynthesis—retained time-of-day patterns (Fig. 2H), although in some cases the magnitude of this difference was altered or did not reach statistical significance.

Next, we determined the effect of disrupted Bmal1-mediated regulation of PC transcription on rhythms in IgA but unexpectedly found that oscillations were retained (Fig. 2I). Because some time-of-day signatures in IgA+ PC transcription were only partly dependent on intrinsic Arntl expression and rhythmicity in IgA secretion was retained, we asked whether IgA secretion into the intestinal lumen could be subject to further circadian regulation at the tissue level. IgA produced by lamina propria–resident PCs requires active transport across the intestinal epithelium by the polymeric Ig receptor (pIgR). However, we failed to detect oscillatory expression of the Pigr gene in small intestinal tissue (Fig. 2I), whereas conditional deletion of Arntl in intestinal epithelial cells (VillinCre × Arntlfl/fl) also failed to perturb rhythmicity in fecal secretory IgA (Fig. 2K). Together, these findings suggest that the IgA+ PC–intrinsic circadian clock is a major contributor to rhythmic transcriptional activity but that rhythms in IgA secretion can persist in the absence of intrinsic clock function, indicating that additional factors may entrain circadian function.

Feeding-associated metabolic cues determine the magnitude and rhythmicity of intestinal IgA responses

Although cell-intrinsic circadian clocks are important for driving oscillatory immune cell activity, additional exogenous signals can act to entrain these circadian rhythms—most notably feeding cues (22, 29). Moreover, emerging evidence suggests that IgA responses are highly sensitive to changes in nutrition and diet (9, 16, 17, 30). To determine whether feeding-associated cues contribute to the entrainment of rhythms in IgA secretion, we used light-tight cabinets on reverse 12-hour light:dark schedules in combination with 6-hour periods of feeding restricted to either the dark phase ( dark-fed) or light phase ( light-fed) (Fig. 3A). Fecal sampling of animals maintained under these conditions at four time points (ZT0, ZT6, ZT12, and ZT18) revealed that dark-fed animals displayed oscillations in fecal IgA similar to those of ad libitum–fed mice (Figs. 1A and 3B), in line with the largely nocturnal feeding patterns of experimentally housed mice. Restriction of food availability to a 6-hour window during the light phase led to a reversal in oscillatory IgA secretion (Fig. 3B and Fig. S4A), indicating that feeding cues act as a key entrainer of IgA secretion in the gastrointestinal tract. Whereas IgA+ PC from dark-fed animals displayed cell-intrinsic time-of-day differences in clock gene expression comparable with those of ad libitum–fed mice, reversal of feeding also reversed clock gene expression patterns (Fig. 3C), which was mirrored in the liver (Fig. S4B). Moreover, reversed feeding similarly inverted time-of-day differences in PC and metabolism-associated genes (Fig. 3D). Conversely, and in line with our findings under ad libitum conditions (Fig. 1 and fig. S1), feeding cue–associated regulation of fecal IgA could not be attributed to alterations in IgA+ PC or IgA+ B cell frequencies in the intestinal tract and associated lymphoid structures (fig. S4, C and D).

Together, these findings suggested that feeding-associated cues, such as dietary-derived nutrients and metabolites, may act upstream to entrain cell-intrinsic clock genes while also acting to regulate cell function through additional mechanisms independent of clock gene expression per se. Because we observed time-of-day differences in a series of metabolic genes despite deletion of Arntl in IgA+ PCs (Fig. 2G), we reasoned that feeding cues may further entrain IgA secretion via effects on PC metabolic activity in concert with clock gene–driven regulation of transcription. We thus hypothesized that alterations in dietary nutritional content may perturb rhythms in IgA secretion. As a proof of concept, we fed mice normal chow or a commercial high-fat diet (HFD) to establish a state of overnutrition and assessed circadian rhythms in IgA secretion at baseline or 2 or 6 weeks later. Animals fed HFD gained a moderate amount of weight over the 6-week period when compared with mice fed normal chow (Fig. S4E), and critically, although postprandial blood glucose was elevated in HFD mice after 6 weeks (Fig. S4F), no signs of impaired glucose tolerance were observed at this time (Fig. S4G). In contrast, mice fed HFD for a prolonged period of 12 weeks began to exhibit elevated fasting glucose levels (Fig. S4G). Fecal IgA levels consistently exhibited circadian oscillations over a 24-hour period in animals fed normal chow and serially sampled at baseline, 2 weeks, and 6 weeks (Fig. 3E). In contrast, whereas the HFD-fed group exhibited a comparable oscillation in fecal IgA at baseline, the same animals began to exhibit dysregulation of oscillatory IgA secretion after 2 weeks on HFD and a complete loss of IgA rhythmicity after 6 weeks (Fig. 3E). However, the overall magnitude of IgA secretion was increased significantly in mice fed HFD for 6 weeks (Fig. 3E), although, as observed previously (31), feeding of mice on an HFD also led to a blunting of the diurnal food intake (fig. S4, H and I). Together, these data further suggest that HFD may disrupt rhythms in IgA.

Cell-intrinsic metabolic activity and nutrient availability have been demonstrated to be critical determinants of PC survival, function, and antibody secretory capacity (14, 15, 32, 33). In line with this concept, we found that small intestinal IgA+ PCs exhibited elevated metabolic activity when compared with either IgA+ B cells or IgD+ B cells derived from the Peyer’s patches (fig. S5, A to J). IgA+ PCs exhibited markedly elevated uptake of the glucose analog 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-d-glucose (2NDBG) (fig. S5, A and B), expressed higher levels of the solute carrier chaperone protein CD98, which functionally endowed cells with enhanced amino acid uptake capacity (fig. S5, C to G), and exhibited elevated intracellular lipid content (fig. S5, H and I). The heightened metabolic activity of IgA+ PCs was further reflected in extracellular flux assays (fig. S5I). Nonetheless, the ex vivo metabolic activity of IgA+ PCs did not significantly differ by time of day (fig. S5, K to N), suggesting that circadian rhythms in IgA+ PC function and IgA secretion were not dictated by diurnal changes in the metabolic capacity of PCs per se. Rather, we hypothesized that changes in nutrient availability—as a result of feeding activity—may act as a rate-limiting factor for antibody secretion by fueling IgA+ PC metabolism and entraining rhythmicity in
concert with the cell-intrinsic clock. In line with this hypothesis, the IgA secretory capacity of sort-purified PCs cultured ex vivo was found to be sensitive to the nutrient content of the culture medium, with an increase in glucose from subphysiological (1 mM) to physiological (9 mM) levels resulting in increased magnitude of IgA secretion (Fig. 3F). Similarly, IgA secretion from cultured PCs was sensitive to the presence of the amino acid leucine in the culture medium (Fig. 3G), although pharmacological inhibition of either amino acid transport (2-amino-2-norbornanecarboxylic acid; BCH) or glycolysis [2-deoxy-D-glucose (2DG)] conversely reduced the magnitude of IgA secretion (Fig. 3H). Together, these findings suggest that feeding-associated cues, potentially through changes in nutrient availability, act to entrain and align oscillations in IgA production and the IgA+ PC transcriptional circadian clock in part by fueling cell-intrinsic metabolic activity.
Reciprocal interactions between host and microbiota determine oscillatory IgA secretion and rhythms in the microbiota to modulate microbial mutualism

IgA is a canonical immune regulator of host-commensal microbe interactions and mutualism, and although a considerable proportion of the microbiota is bound by secretory IgA, the precise effect of IgA on the composition and mutualistic functions of the microbiota has remained incompletely understood. Moreover, emerging evidence suggests that the composition of the microbiota not only exhibits circadian rhythmicity, in part dictated by host immune circuits, but is also likely to provide cues that act to align rhythms in immune responses, highlighting the inherently linked nature of mucosal immune responses (22–25, 27, 34). To first address the role of the microbiota in dictating IgA rhythmicity, we investigated IgA responses in germ-free animals; however, as previously reported, the absence of a microbiota markedly reduces the generation of IgA+ PC in the intestinal lamina propria (fig. S6, A and B). In line with this, IgA levels were markedly reduced in the feces of germ-free animals compared with those of specific pathogen-free (SPF) controls (fig. S6C). To further clarify the role of microbiota in regulating the rhythmicity of the IgA response, we transiently treated SPF mice with a cocktail of antibiotics, which successfully depleted commensal microbes without significantly altering the intestinal IgA+ PC pool (Fig. 4, A and B). Antibiotic treatment led to a clear disruption of normal rhythmicity in fecal IgA, although perturbed IgA production remained statistically significant by JTK_CYCLE analysis, suggesting that IgA rhythms were markedly altered but not absent per se (Fig. 4C).

Although these observations suggest that the microbiota itself is required for homeostatic IgA rhythmicity, we proposed that conversely, IgA responses against the microbiota could in turn regulate oscillations in microbial composition. The dissection of the precise roles of IgA in regulating the commensal microbiota have been hindered by the generation of compensatory IgM responses in both Ighg-knockout mice and Ighg-deficient humans, which bind to a comparable repertoire of commensal bacteria (11, 35–37). To circumvent this issue and determine whether circadian oscillations in intestinal IgA affect the commensal microbiota, we used IgMi mice (38–40), which lack the ability to class switch and secrete antibody yet retain a mature B cell compartment (Fig. 4D). Thus, this model allowed us to study the microbiota in the absence of both secretory IgA and any other mucosal antibody isotypes transported into the intestinal lumen in the absence of IgA that may otherwise fully or partially compensate. As expected, IgMi mice lacked detectable fecal IgA by enzyme-linked immunosorbent assay (ELISA) when compared with littermate control animals (Ctrl) (fig. S6D) and also lacked IgA-bound bacteria as determined by flow cytometry (Fig. 4E and fig. S6E). Next, we serially collected fecal samples from IgMi and littermate control animals over multiple circadian time points and performed 16S ribosomal RNA (rRNA) sequencing. We confirmed time-of-day differences in the total abundance of commensal bacteria as has been previously reported (23–25) but found diurnal oscillations in total microbial abundance to be unaffected in IgMi mice (fig. S6F). Similarly, and in line with previous findings (39), we did not observe any marked changes in the global composition of fecal bacteria at the phylum or genus level when analyzing the microbiota across the mean of all samples by genotype (Fig. 4F; data show average of combined time points). One notable exception was a clear reduction in the abundance of Akkermansia in IgMi mice (Fig. 4F and fig. S6G), suggesting that IgA binding may favor colonization of this mucosal-dwelling microbe. However, a granular analysis by ZT identified rhythms within the commensal microbiota, in line with previous reports (23–25, 27, 34). Consistent with these previous studies, we were able to identify circadian rhythmicity in a number of bacteria genera, including Mucispirillum, Helicobacter, Peptococcaceae, Desulfovibrio, and Bilophila (Fig. 4, G to I, K, and L), whereas other major bacterial genera demonstrated no observable time-of-day differences (fig. S6H). Critically, we identified a signature of rhythmic bacteria that lost circadian rhythmicity in the absence of IgA (Fig. 4, G, K, and L, and fig. S6I), although others retained or gained rhythmicity in IgMi mice (Fig. 4, H and I). To determine whether the loss of bacterial rhythmicity in IgMi mice correlated with direct IgA binding, we first quantified IgA binding of total fecal commensal microbes by flow cytometry but observed no significant differences in the total proportion of the microbiota labeled by IgA over time (fig. S6, M and N). Despite this, by sequencing of IgA-bound versus unbound bacteria (IgA-seq), we identified an enrichment (71%) of oscillatory microbes (red) among bacteria identified to be preferentially IgA bound in control animals (Fig. 4I). Many IgA-bound bacteria also demonstrated a loss of rhythmicity (Fig. 4, G, J and K) or changes in circadian phase (fig. S6K) in the absence of mucosal antibodies. Unexpectedly, although we observed a small subset of oscillatory bacteria that were preferentially enriched in the IgA-negative fraction and unaltered in IgMi mice (fig. S6L), we also detected some bacteria that were not directly bound by IgA yet lost rhythmicity in IgMi mice (Fig. 4L), suggesting that circadian regulation of commensal microbiota is highly complex and potentially subject to reciprocal interactions and competition for niches that alter the relative abundances of microbial species within the community. Thus, we were able to identify oscillations in the abundance of a number of commensal bacteria that were dependent on IgA, the secretion of which is itself regulated in a circadian manner.

Although this finding provides evidence for a circadian role for IgA in regulating the composition of commensal bacteria, the consequences for the mutualistic functions of the microbiota and the mammalian host were unclear. Thus, we further performed shotgun metagenomics on serially sampled fecal bacteria from littermate control mice over five distinct time points (ZT0, ZT6, ZT12, ZT18, and a second ZT0 within the same 24-hour period). Analysis of functional GO terms in wild-type littermates predicted that a significant proportion of predicted bacterial functional pathways undergo circadian oscillation (Fig. 5A). IgMi mice exhibited a near-complete loss of highly oscillatory GO terms when compared with littermate controls (Fig. 5, A and B, and data file S3). Many of the microbial GO terms that were found to be oscillatory in control animals and lost in IgMi mice related to metabolic processes, including Glycolytic Process and Gluconeoegenesis (Fig. 5, B and C, and fig. S7, A and B), suggesting that the presence of IgA may promote rhythmicity in microbial metabolism and liberation of nutrients from the diet. We also identified a small number of GO terms that indicated alterations in basic microbiota biology, including several that in contrast were predicted to gain oscillations in the absence of IgA, including bacterial Flagellum Assembly and Extra-chromosomal Circular DNA (fig. S7C). Next, to determine whether changes in microbial function and metabolic activity altered nutrient availability within the intestine, we performed metabolomics on fecal samples from IgMi mice and littermate controls. We observed
evidence of time-of-day differences in the relative abundance of glucose in the feces over the course of a day, which was blunted in the absence of mucosal antibody (Fig. 5D), and to a lesser extent in short-chain fatty acid availability (Fig. S8A), whereas the availability of succinate exhibited comparable time-of-day differences regardless of the presence or absence of mucosal antibody (Fig. S8A). Despite changes in fecal metabolite levels, we confirmed that IgMi mice retained comparable circadian patterns in food intake (Fig. S8B), suggesting that the differences could not be attributed to changes in feeding behavior. To determine the potential effect of circadian changes in intestinal metabolite availability on the host, we placed mice in metabolic cages (CLAMS) but found no evidence for major dysregulation of whole-body metabolism and energy usage (Fig. S8, C and D). However, we observed perturbed time-of-day differences in circulating glucose in the blood of IgMi mice (Fig. 5E), which mirrored predicted microbial
metabolic activity and glucose abundance in the feces, thus suggesting that circadian IgA regulation of microbial function may modulate time-of-day differences in metabolite availability and/or uptake by the host.

**DISCUSSION**

The complex interplay between the microbiota, intestinal immune system, and diet is increasingly understood to be at the center of a broad range of inflammatory, metabolic, and systemic pathologies—and an increasing driver of morbidity and mortality in the industrialized world. Here, we provide evidence of circadian regulation of a major mucosal immune pathway with critical functions in regulating host-microbiota cross-talk, which we hypothesize may have provided evolutionary benefit by balancing the energetic cost of an intestinal immune response with optimal orchestration of microbial mutualism. Specifically, we report diurnal secretion of IgA—in line with previous observations (41, 42)—and demonstrate feeding as a major cue in aligning rhythms in mucosal antibody secretion. Furthermore, we demonstrate that the absence of IgA significantly affects oscillations in the relative composition and activity of intestinal microbes. Together, our findings suggest that a combination of cell-intrinsic circadian clocks and cell-extrinsic feeding cues entrains rhythms in IgA and, in turn, modulates the commensal microbiota (fig. S9).

Here, we observed the presence of an active transcriptional circadian clock in IgA+ PC, which was unexpectedly largely absent in naïve B cells, indicating that circadian clock rhythmicity may be imprinted during B cell activation or class switching. Nonetheless, we did observe Nr1d1 expression in naïve B cells, which may suggest roles for Rev-erba beyond circadian regulation, as described for other immune cells (43, 44). However, although we observed IgA+ PC–intrinsic oscillations in canonical clock genes and significant disruption of clock gene expression upon deletion of Arntl, rhythmicity in luminal secretory IgA was retained, suggesting that Bmal1 is dispensable for oscillations in IgA secretion and indicating the potential for additional peripheral cues. In line with an emerging body of evidence (45, 46), we identified feeding events as a critical cue to entrain IgA responses and PC rhythmicity. Reversed feeding experiments also similarly perturbed time-of-day differences in metabolic activity and glucose abundance in the feces, thus suggesting that circadian IgA regulation of microbial function may modulate time-of-day differences in metabolite availability and/or uptake by the host.

![Fig. 5. Mucosal antibody regulation of microbiome circadian rhythmicity modulates nutrient and metabolite availability and uptake.](image-url)

- **A** JTK_CYCLE analysis of GO term pathway scores identified by shotgun metagenomics of serially sampled feces of control and IgMi mice over five 6-hour intervals over a circadian day (ZT0, ZT6, ZT12, ZT18, and ZT0); n = 5 per group per time point and representative of a single experiment. Significance cutoff = P < 0.05. **B** Z-score heatmap of average GO term scores identified to be significantly oscillatory in control mice and perturbed in IgMi mice by JTK_CYCLE analysis (P < 0.05) and (C) select exemplar pathways double-plotted. **D** Glucose levels in serially sampled feces of control and IgMi mice over five 6-hour intervals over a circadian day (ZT0, ZT6, ZT12, ZT18, and ZT0); n = 5 per group per time point; representative of a single experiment. **E** Glucose levels in serially sampled blood of control and IgMi mice over five 6-hour intervals over a circadian day (ZT0, ZT6, ZT12, and ZT18; ZT0 double-plotted); n = 8 to 12 per group per time point; representative of data pooled from three independent experiments. P values determined via JTK_CYCLE. All data shown as ±SEM unless otherwise indicated; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
circadian clock genes and other highly rhythmic genes, suggesting that feeding cues may link extrinsic and intrinsic circadian timing in IgA+ PC. Nonetheless, we cannot rule out roles for circadian clock gene regulation independent of Bmal1. For example, other clock components have been reported to retain rhythmicity in the absence of Bmal1, whereas Rev-erba has been attributed clock-independent roles as a transcription factor (43). Moreover, Xbp1, which was also found to be rhythmic in IgA+ PC and entrained by feeding here, has recently been described to induce oscillatory gene expression independent of core clock genes (47).

Our findings provide further evidence for feeding as a critical entraining cue for peripheral circadian rhythms in intestinal immune cells. Elevations in IgA in fecal pellets aligned with periods of feeding in both ad libitum— and reverse-fed mice, although, of note, we observed a lag effect whereby elevations in IgA also persisted into the relative fasting phase, most likely due to the time delay between food ingestion, liberation, and absorption of nutrients, changes in IgA+ PC function, and subsequent detection of secreted IgA after transit of fecal pellets along the intestinal tract. Both long-term undernutrition and chronic overnutrition can alter the generation of IgA responses in the intestinal tract, suggesting a complex interplay between nutrition, circadian rhythms, and mucosal antibody responses and host-commensal mutualism (9, 16, 17, 22, 23, 25, 27, 29, 30). Despite these advances, the precise molecular mechanism through which feeding modulates IgA secretion in the gut remains unclear. One hypothesis is that elevated nutrient availability after feeding acts as a key determinant of the ability of intestinal PCs to fuel enhanced antibody secretion, and in line with this, we demonstrated that altering nutrient or metabolite levels was sufficient to change the magnitude of IgA secretion ex vivo. In addition, the feeding of an HFD disrupted rhythms in IgA, although it should be noted that HFD has marked effects on circadian feeding activity (31), the microbiota, and intestinal inflammation—in addition to altering nutritional content—all of which could feasibly contribute to this phenotype given the interconnected nature of diet, microbiota, and intestinal immunity. However, of note, nutritional cues can be sensed and propagated not only via intracellular metabolic signaling pathways and transcriptional regulators but also by neuronal and hormonal circuits (48). Thus, further studies are required to determine the precise intracellular sensing pathways that connect feeding events with rhythmic IgA+ PC circadian clock-driven transcription and secretory function. Nonetheless, IgA responses and PCs are known to be particularly sensitive to changes in nutrition (9, 16, 17), and studies to decipher the mechanistic basis through which diet alters intestinal IgA production may provide critical insights into the etiology of diet-driven changes in the microbiota that predispose to inflammatory and metabolic disease.

In line with several previous studies, a lack of IgA did not result in a marked global dysbiosis per se, although we observed a loss of Akkermansia species, in line with current understanding that in many cases IgA promotes host mutualism with mucosal-dwelling commensals (49–51). We were able to recapitulate seminal observations made by other groups who reported diurnal oscillations in many of the same commensal microbes, including Mucispirillum, Peptococcaceae, and Streptococcaceae species (23–25, 27, 34). Critically, as in previous studies, these oscillations in bacterial constituents further manifested as time-of-day regulation of commensal function and broader microbial biology—most notably in pathways orchestrating nutrient metabolism, bacterial replication, and pathogenicity (23, 25, 27). The role of IgA in affecting microbial transcription and functional biology—as opposed to composition—has remained relatively poorly understood, although recent advances have begun to delineate the role of IgA binding in regulating microbial metabolic activity, motility, and fitness (10). Here, we demonstrate that the lack of IgA secretion causes a loss in diurnal oscillations at the level of both composition and microbial activity and build upon previous findings in the field to suggest that IgA binding has key roles in modulating bacterial gene expression. For example, we found that the lack of IgA led to a gain in flagellum assembly over the circadian day, supporting findings that IgA binding can suppress bacterial flagellum expression and thus motility (52). One notable observation was the circadian regulation of microbial pathways of glucose metabolism and glucose availability both within the intestine and in circulation—adding to previous findings that IgA may be an important immune pathway in the regulation of glucose metabolism and risk of metabolic disease (16). Despite these advances, the precise mechanism through which oscillations in IgA alter rhythmic microbial composition and function remains to be elucidated but suggests that host-microbiota interactions are highly dynamic over the course of a circadian day. Nonetheless, our findings provoke the hypothesis that circadian immune regulation of the microbiota may act to promote mutualism, metabolite availability, and metabolic health, which together with recent advances (53) suggest that IgA acts to determine host exposure to microbiobally derived metabolites.

Together, our observations complement and expand upon other recent studies that together suggest that circadian regulation may be a common feature of tissue-resident intestinal immune cells that constitutively act to maintain healthy interactions with commensal bacteria (26, 27, 43, 44, 54–57) and that immune pressure may partially imprint rhythmicity on the microbiota itself to confer mutualistic benefits for the host over the daily light-dark cycle, including ensuring energetic and metabolic efficiency aligned with feeding activity. An increasing body of evidence has begun to link lifestyles that disrupt circadian rhythmicity and microbial rhythms with the onset and progression of human inflammatory and metabolic diseases, including type 2 diabetes (58), and thus, an increased understanding of circadian immune regulation will be critical to harness the full potential of the emerging field of circadian medicine (59, 60).

MATERIALS AND METHODS

Study design
The study was designed to determine whether the magnitude, phenotype, or transcriptome of the IgA PC response in the small intestine was subject to diurnal variation. In addition, we aimed to uncover the intrinsic and extrinsic cues that regulate the magnitude of IgA responses over the course of a 24-hour period and to determine the requirement for IgA in contributing to circadian changes in the composition, dynamics, and predicted activity of the intestinal commensal microbiota. All in vivo experiments were performed a minimum of two times, with animal numbers per group indicated in the respective figure captions. All transgenic mouse lines were age- and sex-matched and cohoused with control littersmates within each individual experiment. Mice of different genotypes were randomly assigned to cage and group, and studies were unblinded.
Mice
Age- and sex-matched C57BL/6 mice were purchased from Envigo. 
*Mbi*<sup>Cre</sup> × *Arntl<sup>fl/fl</sup>* mice were backcrossed by K.-M. Toellner (University of Birmingham) and are available via the Jackson Laboratory [*Mbi*<sup>Cre</sup> (61)—strain 020505, originally a gift from M. R. Thum (University of Freiburg), and *Arntl<sup>fl/fl</sup>—strain 007668]. *Villin*<sup>Cre</sup> × *Arntl<sup>fl/fl</sup>* were maintained within the Centre for Biological Timing at the University of Manchester, and *Villin*<sup>Cre</sup> are available via the Jackson Laboratory (strain 004586). IgMi mice were a gift from A. Waisman (Institute of Molecular Biology, Mainz) and are available subject to collaborative agreement. All transgenic mouse experiments were performed using cohoused littermates, of mixed genotype, and under SPF conditions with ad lib feeding at 12-hour light-dark cycle at the University of Manchester, United Kingdom, unless otherwise specified. In some cases, mice received irradiated HFD (Research Diets, D12492i; 60% kcal from fat) ad libitum for up to 12 weeks. Where indicated, experimental cages were placed in controlled light-tight cabinets under opposing 12-hour light-dark cycles to facilitate investigation of circadian rhythms. In some experiments, mice were placed in bespoke housing for the measurement of metabolic readouts and feeding as detailed below. Germ-free mice were bred and maintained in the University of Manchester Axenic and Gnotobiotic Facility. All other animal experiments were performed under SPF conditions in single ventilated cage conditions and under license of the U.K. Home Office and under approved protocols at the University of Manchester.

Tissue processing
Small intestinal lamina propria lymphocyte preparations were prepared by opening longitudinally and removing the Peyers patches, associated fat, and luminal content by gently shaking in cold phosphate-buffered saline (PBS). Epithelial cells and intraepithelial lymphocytes were removed by shaking tissues in stripping buffer (1 mM EDTA, 1 mM dithiothreitol, and 5% fetal calf serum [FCS]) for two rounds of 20 min at 37°C. Lamina propria lymphocytes were isolated by digesting the remaining tissue in collagenase D (1 mg/ml; Roche) and deoxyribonuclease I (20 μg/ml; Sigma-Aldrich) for 45 min at 37°C. Liberated cells were then extracted by passing the tissue and supernatant over a 70-μm nylon filter and centrifuged to isolate lamina propria lymphocytes. Isolated Perys patches were processed by passing them through a 70-μm nylon filter. In a small number of cases, Perys patches were retained during intestinal tissue digest to facilitate concurrent analysis of tissue-resident PCs and B cell subsets.

Flow cytometry
Single-cell preparations were stained with antibodies against surface or intracellular markers as indicated in table S1. Specific conjugates used are indicated within the figures. Dead cells were excluded from analysis using LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies). Intracellular staining was performed using a FoxP3 fixation/permeabilization kit (Thermo Fisher Scientific). Samples were acquired using a BD Fortessa cytometer and analyzed with FlowJo (Tree Star).

Bacterial flow cytometry
Feces were collected in Fast Prep Lysing Matrix A tubes (MP Biomedicals), resuspended in 1 ml of PBS per 100 mg of fecal material, and incubated at 4°C for 20 min. Bacterial suspensions were resuspended in a final volume of 2 ml of PBS and incubated at 4°C for 20 min. Samples were homogenized in a FastPrep-24 Tissue homogenizer (MP Biomedicals) for 30 s. After homogenization, samples were centrifuged at 50g for 15 min at 4°C to remove debris, and the bacteria-containing supernatant was transferred through 70-μm filters into a new tube. Bacteria were washed in fluorescence-activated cell sorting (FACS) buffer (PBS, 2% FCS, and 5 mM EDTA) and pelleted at 8000g for 5 min. For flow cytometry, bacterial pellets were resuspended in 100 μl of FACS buffer containing SYTO 9 green fluorescent nucleic stain (Life Technologies) (10 μM), incubated at 4°C for 15 min, and subsequently stained with an anti-mouse IgA-PE (phycoerythrin) antibody (1 μg/ml) (clone mA-6E1, eBioscience) for 30 min at 4°C. Samples were thoroughly washed and acquired on a BD Fortessa flow cytometer.

Cell sorting and ex vivo culture assays
Ex vivo culture assays were performed on sort-purified IgA* PCs, IgA* or IgD* B cells, or other control populations isolated from the small intestines and/or Peyers patches of female C57BL/6 mice unless otherwise indicated. Kynurenine uptake was assessed as previously reported (62). Briefly, after surface antibody staining, 2 × 10<sup>6</sup> cells were resuspended in 200 μl of warmed Hank’s balanced salt solution (HBSS) (Sigma-Aldrich, UK), and 100 μl of HBSS, BCH (40 mM, in HBSS), or leucine (20 mM, in HBSS) was added to appropriate samples. Kynurenine (800 μM, in HBSS) was then added, and uptake was subsequently stopped after 4 min by adding 125 μl of 4% paraformaldehyde for 30 min at room temperature in the dark. After fixation, cells were washed twice in HBSS and then resuspended in HBSS before acquisition on the flow cytometer. For assessment of 2-NBDG uptake in vitro, 1 × 10<sup>6</sup> small intestinal cells were cultured in glucose-free Dulbecco’s modified Eagle’s medium (DMEM) medium (Agilent, USA) supplemented with 2 mM L-glutamine and 100 μM 2-NBDG (Thermo Fisher Scientific, USA) for 10 min at 37°C. Surface antibody staining of samples was then performed, and acquisition of samples on the flow cytometer was undertaken within 2 hours. For assessment of lipid accumulation within cells in vitro, 1 × 10<sup>6</sup> small intestinal cells were cultured in glucose-free DMEM medium (Agilent, USA) supplemented with 2 mM L-glutamine and LipidTOX (Thermo Fisher Scientific, USA) for 30 min at 37°C. Cells were then washed, surface antibody staining of samples was performed, and acquisition of samples on the flow cytometer was undertaken within 2 hours.

Antibiotic treatment
Mice were treated for a total of 6 days with an antibiotic cocktail containing ampicillin (1 mg/ml), neomycin (1 mg/ml), gentamicin (1 mg/ml), metronidazole (0.25 mg/ml), and vancomycin (0.5 mg/ml) in water supplemented with artificial sweetener tablets for palatability. After 3 days of antibiotics, depletion of the microbiota was confirmed by counting colony-forming units (CFUs) of homogenized fecal pellet supernatants on Brain Heart Infusion (BHI) medium (CM1134, Thermo Fisher Scientific) plates cultured under both aerobic conditions at 37°C for 48 hours or incubated in Wilkens-Chalgren anaerobe medium enriched with 10% horse blood (CM1135, Thermo Fisher Scientific) under anaerobic conditions in a Whitley MG500 anaerobic incubator with 10% (v/v) H<sub>2</sub>, 10% CO<sub>2</sub>, and 80% N<sub>2</sub> at 37°C for 48 hours. Feces of antibiotic-
treated mice were subsequently collected over four circadian time points beginning on day 4 to measure IgA.

**Enzyme-linked immunosorbent assay**

Mouse fecal IgA titers were measured using the Mouse IgA ELISA Quantitation Set (Bethyl Laboratories) following the manufacturer’s instructions. Fecal samples were serially diluted, and optimal dilutions and concentration were determined on the basis of a standard curve. For core datasets, an additional bicinchoninic acid (BCA) assay [Pierce Coomassie Plus (Bradford) Assay Kit, Thermo Fisher Scientific] was performed on fecal extracts to measure total protein, and IgA concentrations were normalized.

**Metabolic inhibitor assays**

Sort-purified IgA+ PCs isolated from the small intestinal lamina propria were incubated (10⁴ cells per well) in either leucine-free medium (US Biological, USA) or glucose-free medium (Gibco, UK), with interleukin-6 (IL-6) (10 ng/ml) (PeproTech, USA) and B-cell activating facor (BAFF) (200 ng/ml) (BioLegend, UK), supplemented with differing concentrations of leucine or glucose (both Sigma-Aldrich, UK). To determine the effects of inhibiting nutrient uptake or metabolic signaling on IgA secretion, we cultured sort-purified IgA+ PCs isolated from the small intestinal lamina propria (10⁴ cells per well) as above with or without the addition of metabolic inhibitors including pp242 (500 nM), BCH (10 mM), and 2DG (1 mM) (all Sigma-Aldrich, UK). Cells were incubated for 16 hours at 37°C, after which culture supernatants were removed and IgA concentrations were determined by ELISA. Cell viability was determined under different culturing conditions, using either a hemocytometer or a flow cytometer.

**Extracellular flux analysis**

Extracellular flux analysis (Agilent, USA) was performed with replicates of 150,000 sort-purified IgA+ PCs isolated from small intestinal lamina propria or IgD+ B cells isolated from Peyer’s patches. Cells were adhered to each well of the Seahorse plate (Seahorse/Agilent, USA) using Cell-Tak (Corning, USA). Cells were rested in Seahorse medium (glucose-free DMEM) at 37°C without CO₂ for at least 30 minutes before the run. For the test, Seahorse XF medium was supplemented with 2 mM l-glutamine (Sigma-Aldrich, UK), and pH was adjusted to 7.35 ± 0.05 (at 37°C). Glucose (10 mM final concentration) (Thermo Fisher Scientific, USA), oligomycin (1 μM final concentration) (Sigma-Aldrich, UK), and 2DG (100 mM final concentration) (Sigma-Aldrich) were added to individual wells to complete this assay.

**Metabolic and physiological monitoring**

To assess metabolic gas exchange, we housed mice individually in indirect calorimetry cages (CLAMS cages, Columbus Instruments). Mice previously maintained on a controlled light-dark light cycle were acclimatized to the cages for two 24-hour cycles, and oxygen consumption and carbon dioxide production were recorded at 10-min intervals for at least a further three consecutive 24-hour light-dark cycles. Respiratory exchange ratio was derived from these measurements (VCO₂/VO₂), as was energy expenditure. For measurement of food intake, genotype-matched cohoused mice were placed in the Sable System for a full week on a controlled 24-hour light-dark cycle. After a 2-day acclimatization period, food intake was measured for at least three consecutive 24-hour light-dark cycles.

**Reverse transcription polymerase chain reaction**

Total RNA was purified using the RNeasy Micro Kit (Qiagen), and complementary DNA (cDNA) was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR (qPCR) was performed with the real-time PCR StepOnePlus system (Applied Biosystems). Nrl1d1 (Mm01304569_m1), Hk2 (Mm00443385_m1), Hmgs (Mm01034569_m1), and Xbp1 (Mm00457358_m1) were detected with commercial TaqMan probe assays (Applied Biosystems). The remaining assays were performed with either TaqMan-based chemistry (Applied Biosystems) or LightCycler 480 SYBR Green I Master Mix (Roche) using the primers and probes detailed in table S2.

**Bulk RNA-seq**

RNA was isolated from sort-purified cells, as above, and library preparation and bulk RNA-seq were performed commercially with Novogene (UK) Company Ltd.Briefly, normalized RNA was used to generate libraries using the NEB Next Ultra RNA Library Prep Kit (Illumina). Indices were included to multiplex samples, and mRNA was purified from total RNA using poly-thymidine oligo-attached magnetic beads. After fragmentation, the first-strand cDNA was synthesized using random hexamer primers, followed by second-strand cDNA synthesis. After end repair, A-tailing, adapter ligation, and size selection, libraries were further amplified and purified, and insert size was validated on Agilent 2100 and quantified using qPCR. Libraries were then sequenced on an Illumina NovaSeq 6000 S4 flow cell with PE150 according to results from library quality control and expected data volume. RNA-seq data are available via the Gene Expression Omnibus (GEO) repository (accession numbers GSE175637 and GSE175609).

**Bacterial 16S PCR**

Bacteria from fecal pellets were extracted and normalized to fecal weight, and DNA was isolated using the DNeasy PowerLyzer PowerSoil Kit (Qiagen) according to the manufacturer’s instructions. Bacterial DNA samples were amplified using the following primers: universal 16S, 5'-GCAGGCCTAACACATGCAAGTC-3' (forward) and 5'-CTGCTGCTCCCTGGTAGAGT-3' (reverse). Real-time PCR was performed with the StepOnePlus Real-Time PCR system using SYBR Green Master Mix (Applied Biosystems). Bacterial copy number was calculated using a standard curve of known CFU and DNA concentrations from *Staphylococcus aureus* cultures.

**16S rRNA-seq**

Bacterial DNA from fecal bacteria was isolated using the PowerSoil DNA Isolation Kit (Qiagen, The Netherlands) according to the manufacturer’s instructions. Preamplification of the V3V4 region of 16S rRNA was performed by PCR in triplicate using 2xKAPA HiFi Hot Start ReadyMix (Roche) using primer pairs containing adaptor sequences as follows: 16S amplicon PCR, 5'-TCGTCGACGCTCAAGATGTATAGAAGACAGGC-TACGGGNNGGCGAGAG (forward) and 5'-GCTCCTGGGGCTCGGAGATGTATAGAAGACAGGC-TACHVGGTAGTACCTCC (reverse). After this, AMPure XP beads (Thermo Fisher Scientific) were used to purify the 16S V3V4 amplicon away from free primers and primer dimer species according to the manufacturer’s protocol. Illumina sequencing adapters were then attached using the Nextera XT Index Kit.
Shotgun metagenomics

Shotgun metagenomics was performed commercially by CosmoID. Briefly, microbial DNA was extracted from fecal pellets and quantified using a Qubit 4 fluorometer and the HS Assay Kit (Thermo Fisher Scientific). DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit and Nextera Index Kit (Illumina) following the manufacturer’s protocol with minor modifications. The standard protocol was used for a total DNA input of 1 ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Combinatorial dual indexes were added to each sample, followed by 12 cycles of PCR amplification. DNA libraries were then purified using AMPure magnetic beads (Beckman Coulter) and eluted in Qiagen EB buffer. DNA libraries were requantified and pooled together for sequencing via the Illumina HiSeq X. Raw reads from metagenomic samples were analyzed by CosmoID metagenomic software (CosmoID Inc., Rockville, MD, USA) to identify microbes to the strain level, and a high-performance data mining k-mer algorithm was used alongside highly curated dynamic comparator databases to rapidly disambiguate short reads into related genomes and genes.

Functional profiling of shotgun metagenomic data

After the initial quality control, adapter trimming and preprocessing of metagenomic sequencing reads were performed using BBduk. The quality-controlled reads were then subjected to a translated search using Diamond against a comprehensive and nonredundant protein sequence database, UniRef 90. The mapping of metagenomic reads to gene sequences was weighted by mapping quality, coverage, and gene sequence length to estimate community-wide weighted gene family abundances. Gene families were then annotated to MetaCyc reactions (Metabolic Enzymes) to reconstruct and quantify MetaCyc metabolic pathways in the community. Furthermore, the UniRef_90 gene families were regrouped to GO terms to generate an overview of community function. To facilitate comparisons across multiple samples with different sequencing depths, we normalized the abundance values using total-sum scaling (TSS) normalization to produce “copies per million” units.

Bioinformatics

Where indicated, bioinformatic analyses of data were performed via commercial platforms. For analysis of bulk RNA-seq data, differential gene expression analyses were performed in R (version 4.0.2) using RStudio version 1.2.5033 (RStudio Inc.). Raw nonnormalized counts were imported into R and subsequently analyzed using the DESeq2 package (63) using the default pipeline. Genes with a total of fewer than 10 counts across all samples were removed, and normalization was calculated using the DESeq() function with default parameters for estimating size factors and dispersions. Differential expression was then calculated using the results() function with the default parameters. Genes with a significance value of less than 0.01 after correction for multiple comparisons using the Benjamini-Hochberg method were defined as “differentially expressed” and taken forward for further analysis. In some cases, heatmaps were generated from normalized counts using the counts (normalized = TRUE) function, followed by scaling and centering. Hierarchical clustering of genes was then computed using the ComplexHeatmap package (64). In other cases, clustering and normalized counts were then exported to Excel and plotted in GraphPad Prism.

Metabolomics

The metabolic profiles of fecal samples were measured using 1H nuclear magnetic resonance (NMR) spectroscopy as previously described (65). Briefly, fecal samples (30 mg) were defrosted and combined with 600 μl of water and zirconium beads (0.45 g). Samples were homogenized with a Precellys 24 instrument (45 s per cycle, speed 6500, two cycles) and spun at 14,000g for 10 min. The supernatants (400 μl) were combined with 250 μl of phosphate buffer [pH 7.4, 100% D2O, 3 mM NaNO3, and 1 mM 3-(trimethyl-silyl)-[2,2,3,3,3H4]-propionic acid (TSP) for the chemical shift reference at δ0.0] before centrifugation at 14,000g for 10 min and then transferred to 5-mm NMR tubes for analysis on a Bruker 700 MHz spectrometer equipped with a cryoprobe (Bruker BioSpin, Karlsruhe, Germany) operating at 300 K. 1H NMR spectra were acquired for each sample using a standard one-dimensional pulse sequence using the first increment of the nuclear Overhauser effect pulse sequence for water suppression as previously described (66). Raw spectra were phased, baseline-corrected, and calibrated to TSP using Topspin 3.2 (Bruker BioSpin) and then digitized in a MATLAB environment (version 2018, MathWorks Inc., USA) using in-house scripts. Redundant spectral regions (related to water and TSP resonance) were removed, and the spectral data were manually aligned and normalized to the probabilistic quotient using in-house MATLAB scripts. The peak integrals (relating to absolute abundance) for metabolites of interest were calculated for each sample.

Statistical analyses

Statistical analysis of rhythmicity was calculated via JTK_CYCLE analysis (67) of double-plotted datasets using an established R pipeline. Datasets were tested for normality using a Shapiro-Wilks test to determine Gaussian distribution. For two-way comparisons, statistics were performed by t test or Mann-Whitney test as otherwise indicated in the figure legends. For comparisons of three or more groups, or select circadian datasets, a Kruskal-Wallis test, one-way analysis of variance (ANOVA), or two-way ANOVA was performed as appropriate and as indicated in the figure legends. Values for statistical analyses are additionally reported in the raw data file (data file S4). Data are shown as means ± SE.

Supplementary Materials

This PDF file includes:
Figs. S1 to S9
Tables S1 and S2

Other Supplementary Material for this manuscript includes the following:
Data files S1 to S4
MDAR Reproducibility Checklist

View/request a protocol for this paper from Bio-protocol.

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Penny et al., Sci. Immunol. 7, eabk2541 (2022) 2 September 2022