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***Akkermansia muciniphila* induces intestinal adaptive immune responses during homeostasis**

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Abstract

Intestinal adaptive immune responses influence host health, yet only a small number of intestinal species have been identified that induce cognate adaptive immune responses during homeostasis. Here, we show that *Akkermansia muciniphila*, an intestinal bacterium associated with systemic effects on host metabolism and PD-1 checkpoint immunotherapy, induces IgG1 antibodies and antigen-specific T cell responses in mice. Unlike previously characterized mucosal responses, T cell responses to *A. muciniphila* are limited to T follicular helper cells in a gnotobiotic setting, without significant induction of other T helper fates or migration to the lamina propria. However, *A. muciniphila*-specific responses are context-dependent, and adopt other fates in conventional

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Data and materials availability: 16S rDNA sequencing raw data can be accessed at “ncbi.nlm.nih.gov” with the BioProject accession number PRJNA514379 and BioSample accession number SAMN10724098. All other data needed to evaluate the conclusions in this paper are available in the main text or the supplementary materials.

mice. These findings suggest that, during homeostasis, contextual signals influence T cell responses to the microbiota and modulate host immune function.

One-Sentence Summary:

Intestinal immunity to *A. muciniphila* consists of IgG1 antibodies and T_{FH} cells but can be skewed to diverse T cell fates by additional contextual cues.

Adaptive immune cells are critical contributors to tissue homeostasis in the intestine, with B cell-derived IgA playing a major role in establishing barrier function (1). T cell-independent (TI) IgA recognizes a broad fraction of intestinal microbes. In contrast, only a few species that induce T cell-dependent (TD) IgA have been identified (2–4). Until recently, intestinal IgG antibody responses were only thought to occur in the context of mucosal barrier disruption (5) or in response to enteric pathogens (6) and certain pathobionts that breach the intestinal barrier (7). However, recent work has revealed that mice also generate broadly reactive TI anti-commensal IgG2b and IgG3 in a largely Toll-like receptor (TLR) 2- and TLR4-dependent manner (8).

Despite the abundance of foreign commensal antigens and the high frequency of effector and memory T cells within the intestine, only a small number of intestinal species have been identified that induce antigen-specific T cell responses during homeostasis (1, 9, 10). Inappropriate T cell responses against the microbiota are believed to contribute to the pathogenesis of inflammatory bowel disease (IBD) (11, 12). However, anti-commensal effector T cell responses have also been hypothesized to provide bystander protection in the context of enteric infections (13). Thus, understanding which commensal species induce cognate T cell responses, the signals that mediate their induction and regulation, and their effects on host physiology remain important goals in the field. Here, we describe an anti-commensal TD IgG1 response and use it to identify and characterize a commensal species that induces T cell responses during homeostasis.

We examined serum antibody binding to intact commensal bacteria by staining fecal samples with paired mouse sera and isotype-specific secondary antibodies as previously described (8). Surprisingly, comparing wild-type (WT) and T cell-deficient (*Tcrb*^{-/-}) mice revealed that mice mount a microbiota-reactive IgG1 antibody response entirely dependent on αβ T cells (Fig. 1A, B, Fig. S1A–C), whereas anti-commensal IgG2b, IgG3, and IgA are induced in a TI manner as previously reported (Fig. S1A) (4, 8, 14). The fraction of commensals bound by TD IgG1 was approximately 10% (Fig. 1B), far less than the percentage recognized by IgA, IgG2b, and IgG3 (Fig. S1A). Thus, mice produced IgG1 that appeared to recognize only a subset of microbes in the intestine, in contrast to the polyreactive TI antibodies that bind diverse bacteria (8, 15). T cell-sufficient (*Tcrb*^{+/-}) and T cell-deficient (*Tcrb*^{-/-}) cohoused littermates were compared to control for potential differences in the microbiota between these strains. This revealed an anti-commensal IgG1 response only in T cell-sufficient mice (Fig. S1B). Moreover, analysis of mice from multiple vendors and of different genetic backgrounds demonstrated that this IgG1 response was a general feature of healthy mice (Fig. 1C).

To identify the commensal species targeted by this humoral response, we sorted IgG1-bound and -unbound populations from fecal samples stained with sera from corresponding mice (Fig. S2A, B) and performed 16S rDNA sequencing on the resulting fractions (IgG1-Seq). Two operational taxonomic units (OTUs) were significantly enriched in the IgG1-positive fractions compared to the IgG1-negative fractions (Fig. 1D and Fig. S2C, D). These OTUs correspond to the *Akkermansia* genus (OTU2) and the Bacteroides S24–7 family (OTU63). Bacteroides S24–7 comprises a poorly characterized family of mouse intestinal microbes (16). *Akkermansia* is a genus of commensals in the Verrucomicrobia phylum, which until recently, only contained one member, *Akkermansia muciniphila* (17). *A. muciniphila* can degrade mucin (17) and is an abundant member of the human intestinal microbiota (18). Colonization with *A. muciniphila* has been reported to have protective effects in diet-induced obesity (19, 20), to promote mucosal wound healing (21), and to increase anti-tumor responses during anti-PD-1 immunotherapy (22). The mechanisms by which *A. muciniphila* mediates these diverse effects remain poorly understood, as little is known about host sensing of this bacterium. Therefore, based on the ability of *A. muciniphila* to induce TD IgG1 responses and its reported effects on host physiology, we sought to characterize the immune response to *A. muciniphila*.

We isolated *A. muciniphila* from mice in our colony by plating feces on selective media (17). We then used bacterial flow cytometric analysis to confirm the presence of *A. muciniphila*-specific IgG1 antibodies in the sera of mice that harbored *A. muciniphila* at steady state (Fig. 2A). These IgG1 antibody responses to *A. muciniphila* could consist of pre-existing natural polyreactive specificities or antigen-specific responses. To discriminate between these possibilities, we identified C57BL/6 mice lacking *A. muciniphila* in their microbiota (Fig. 2B). Comparing IgG1 antibody responses between *A. muciniphila*-negative and *A. muciniphila*-positive mice confirmed that the induction of *A. muciniphila*-specific serum IgG1 responses required colonization with *A. muciniphila* as well as T cells (Fig. 2A–C). *A. muciniphila*-positive mice also mounted serum *A. muciniphila*-specific TD IgA responses (Fig. S3B). Moreover, de novo colonization of *A. muciniphila*-negative mice by oral gavage was sufficient to induce *A. muciniphila*-specific IgG1 antibodies (Fig. 2D–F and Fig. S3C). Thus, IgG1 responses to *A. muciniphila* are not derived from pre-existing cross-reactive specificities. Rather, mice mount an antigen-specific TD IgG1 antibody response upon *A. muciniphila* colonization.

We noted that titers of serum IgG1 responses against *A. muciniphila* were variable across *A. muciniphila*-positive mice. A small number of mice lacked *A. muciniphila*-specific IgG1 altogether, despite similar colonization (Fig. 2B, C). One explanation for this variability is that variation within intestinal microbial communities may alter the response to *A. muciniphila*. Indeed, previous studies have shown that intestinal infection or inflammation can lead to altered bystander responses against commensal microbes (23). To overcome such complications, we established a defined gnotobiotic system to examine whether direct engagement of the mucosal immune system by *A. muciniphila* underlies the TD IgG1 response. To this end, we introduced *A. muciniphila* into gnotobiotic C57BL/6 mice colonized with altered Schaedler flora (ASF) (24, 25) to generate two mouse colonies with identical microbiota, except for the presence of *A. muciniphila* in the ASF+Akk colony. *A. muciniphila* colonized ASF+Akk mice to high levels and was vertically transmitted (Fig.

2G, and Fig. S3D). We restricted all of our analyses to progeny (or progeny of progeny) of ASF+Akk mice that acquired *A. muciniphila* via vertical transmission. Mice colonized with the ASF+Akk flora, but not the ASF flora alone, mounted IgG1 and IgA antibody responses specific for *A. muciniphila* which, in contrast to conventional mice, had very consistent titers between mice (Fig. 2H, I). Thus, *A. muciniphila* directly engages the immune system to induce TD IgG1 and IgA.

In order to explore T cell responses to *A. muciniphila*, we expanded *A. muciniphila*-specific T cell lines from intestinal tissues of *A. muciniphila*-colonized mice and generated T cell hybridomas via fusion with BWZ.36 cells (26) (Fig. S4A, B). We identified the antigens recognized by two of these hybridomas (124-2 and 168-H10) by screening an *A. muciniphila* genomic expression library for clones that stimulated each T cell hybridoma (Fig. S4C–E). The 124-2 hybridoma recognized a peptide derived from Amuc_RS03735, an outer membrane autotransporter domain-containing protein. Next, we generated *A. muciniphila*-specific TCR transgenic mice (Amuc124), using the TCR α and TCR β chains from the 124-2 T cell hybridoma (Fig. S4A and S5A, B). Importantly, we maintained Amuc124 mice free of *A. muciniphila* colonization for all subsequent experiments.

We sought to track the T cell response to *A. muciniphila* by performing low-frequency adoptive transfers of naïve congenically marked (Thy1.1) Amuc124 CD4⁺ T cells into ASF and ASF+Akk mice (Fig. S6A). *A. muciniphila*-specific T cells expanded in ASF+Akk mice but were undetectable in ASF mice, indicating that *A. muciniphila* antigens are presented under homeostatic conditions (Fig. 3A, B and Fig. S6A, B). Interestingly, Amuc124 T cells localized to the Peyer's patches (PP) and to the mesenteric lymph nodes (mLN), but very few cells were found in the large intestine or small intestine lamina propria (LILP or SILP) (Fig. 3A, B and Fig. S6A, B). The majority of transferred T cells expressed the T follicular helper (T_{FH}) cell markers PD-1, Bcl6, and CXCR5 (Fig. 3C–E, Fig. S6C, D), with a small percentage adopting regulatory T (T_{reg}) cell markers (FOXP3). The small number of T cells detectable in the lamina propria were also skewed towards a T_{FH} cell phenotype, most likely indicating that secondary or tertiary lymphoid tissues were not completely excluded from these preparations (Fig. 3F). Transferred *Rag1*^{+/+} or a *Rag1*^{-/-} Amuc124 T cells yielded similar results (Fig. S7A–D). Surprisingly, transferred T cells expressing markers for T helper (T_H)1, T_H2, or T_H17 cells (T-bet, GATA3, or ROR γ t, respectively) were not detected at appreciable numbers (Fig. S6D).

In order to track endogenous *A. muciniphila*-specific T cells, we generated I-A^b tetramers loaded with the peptide TLYIGSGAILS from the outer membrane protein Amuc_RS03735 (Am3735–1) and confirmed that these tetramers bound Amuc124 TCR transgenic T cells (Fig. S4F). Tetramer-positive, *A. muciniphila*-specific T cells were identified in the PP of ASF+Akk but not ASF mice (Fig. 3G, H), consistent with the expansion of Amuc124 T cells after transfer into ASF+Akk mice. Moreover, the endogenous *A. muciniphila*-specific T cells identified by tetramer staining also expressed T_{FH} cell markers (Fig. 3I, J). An independent tetramer with a different *Akkermansia* epitope from Amuc_RS03740 (Am3740–1) yielded very similar results (Fig. 3H–J). Finally, we probed all gut-associated lymphoid tissue (LILP, PP, and SILP) and mLN with both tetramers. *A. muciniphila*-specific T cells were detected in the PP but not in the lamina propria (Fig. 3K), which confirmed

results obtained with TCR transgenic T cell transfers. Thus, *A. muciniphila* induces antigen-specific T cell responses in the intestine that manifest primarily as T_{FH} cells in the Peyer's patches.

Finally, we returned to SPF mice, where we had observed greater variability in the *A. muciniphila* IgG1 response, and characterized the T cell response to *A. muciniphila* in the context of a conventional microbiota. Similar to our findings for the ASF system, transferred Amuc124 transgenic T cells expanded and localized to the PP in *A. muciniphila*-positive mice but were undetectable in *A. muciniphila*-negative mice (Fig. 4A, B and Fig. S8A). The majority of transferred T cells in the Peyer's patches also adopted T_{FH} cell markers (Fig. 4C and Fig. S8B). Thus, *A. muciniphila* also induces a T_{FH} cell response in the context of a complex microbiota. However, unlike the ASF+Akk system, greater numbers of transferred T cells were detected in the intestinal LP of *A. muciniphila*-positive mice (Fig. 4B), some of which adopted markers consistent with pro-inflammatory T cell fates (Fig. 4D, E and Fig. S8C–E). In line with the variable *A. muciniphila*-specific IgG1 titers observed in SPF mice (Fig. 2C), some cohorts of SPF Akk⁺ mice lacked significant T cell activation and proliferation after transfer, despite the presence of *A. muciniphila*. Thus, T cell responses to *A. muciniphila* appear to be context-dependent, resulting in the induction of other CD4 T effector fates in addition to T_{FH} cells in certain conditions. Interestingly, and in contrast to what has been described for SFB and *Helicobacter* spp. (9, 10, 27), T cell responses to *A. muciniphila* in SPF mice were mixed between different CD4 T cell fates (Fig. S8E).

Commensal-specific TD antibodies were previously thought to be restricted to IgA responses specific for a small subset of commensal species (2). Our work reveals that such TD antibodies also include IgG1, which recognizes a small subset of commensals, including *A. muciniphila*. These bacteria may share certain features, such as proximity to the intestinal epithelium, which may increase their potential to cause disease during barrier disruption. Indeed, systemic antibodies specific for commensal bacteria have been reported to protect against gut-derived septicemia (7, 28), and *A. muciniphila* can promote disease in certain immunodeficient settings (29).

Very few commensal antigens have been identified to date that lead to antigen-specific T cell responses in the gut during homeostasis. SFB and *Helicobacter* spp. both elicit very defined responses. SFB induce RORγt⁺ T_H17 cells both in conventional and mono-colonized mice (27), whereas *Helicobacter* spp. induce RORγt⁺ FOXP3⁺ iT_{reg} cells in the large intestine lamina propria (9, 10). T_{FH} cells comprised a small proportion of SFB and *Helicobacter*-specific T cells, but these were in the context of T_H17- or T_{reg} cell-dominated responses, respectively (10). T_{FH} cells in the intestine have been suggested to differentiate from either T_H17 or T_{reg} cells (30–32). In contrast, the ASF+Akk system produced commensal-specific T cell responses dominated by the induction of T_{FH} cells, with very few T_{reg}, T_H1, T_H2, or T_H17 cells. Thus, commensal-specific T_{FH} responses can occur in the absence of a primary CD4 T cell response of a different fate and may differentiate from naïve commensal-specific T cells in the mesenteric lymph nodes or Peyer's patches. Consequently, *A. muciniphila* appears to engage the mucosal immune system in a manner distinct from previously described T cell-activating commensal bacteria. This commensal-specific T_{FH} response appears in conjunction with robust anti-commensal TD IgG1 and IgA. Surprisingly, in the

context of a conventional microbiota, and in addition to the induction of T_{FH} cells, *A. muciniphila* can induce CD4 T cells of other fates that home to the lamina propria. Together, these results support the hypothesis that T cell responses against commensals can be context-dependent, not just in the setting of acute gastrointestinal infection or inflammation (23), but also during homeostasis. Interactions with certain microbes may change the localization or function of *A. muciniphila*, or signals provided by other microbes may shape the immune response against this commensal bacterium.

Prior work has established that *A. muciniphila* mediates effects on host metabolism (19, 20) and can influence the efficacy of anti-PD-1-based immunotherapy against cancer (22). The mechanisms for these effects remain poorly understood, but both appear to be immune-mediated and correlate with type 1 immunity. In particular, responses to anti-PD-1-based immunotherapy in humans correlated with interferon gamma production by peripheral T cells when incubated with *A. muciniphila* antigens in vitro (22). Interestingly, not all patients generated type 1 responses against *A. muciniphila*. Our results provide a potential explanation for this varied response, and suggest that differential skewing of *A. muciniphila*-specific T cell responses in individuals, due to differences in microbiota composition, or other environmental signals, may have profound systemic effects. Defining the mechanisms by which these anti-commensal T cell responses can be skewed towards different fates is an important goal with clear clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Mice generate anti-commensal IgG1 antibodies during homeostasis

(A) Representative IgG1 flow cytometric analysis of fecal microbiota with sera from WT and T cell-deficient (*Tcrb*^{-/-}) mice. Feces and sera originated from the same mouse (paired serum), except when using antibody-deficient (*Ighm*^{-/-}) serum as a negative staining control. SYBR-green labels a fraction of the microbiota, ensuring that SYBR^{hi} events are bacteria, whereas some of the SYBR^{lo} events are also commensals that are less permeable to the dye (8).

(B) IgG1 microbiota flow cytometric analysis, compiled from eight independent experiments. All mice were housed at UC Berkeley. WT n=63, *Tcrb*^{-/-} n=35 in total.

(C) IgG1 microbiota flow cytometric analysis with paired feces and sera from mice of the indicated genetic backgrounds and vivaria. Balb/c from Jackson Laboratories. Jax B6 and Tac B6: C57BL/6 from Jackson Laboratories or Taconic Biosciences, respectively. SW: Swiss Webster from Taconic Biosciences. n=5 mice per group. Data are representative of two independent experiments.

(D) Results from sorting and 16S rDNA sequencing of IgG1-bound and unbound fractions (n=12 mice). Graph depicts the average log2 ratio of abundances between both fractions for each individual OTU and the corresponding q-value. Data are representative of two independent experiments.

Each symbol represents a mouse (B, C) or an OTU (D). Error bars represent mean $\pm$ SD. Gates on flow cytometry plots show mean $\pm$ SEM. *p*-values were calculated by a Kruskal–Wallis test followed by Dunn’s multiple comparisons (B) or by Paired ratio Student’s *t*-test followed by Benjamini, Krieger and Yekutieli’s two-stage false discovery rate (FDR) to correct for multiple comparisons, with an FDR (Q) of 0.01 (D).

Fig. 2. *Akkermansia muciniphila* is necessary and sufficient to induce cognate *A. muciniphila*-specific IgG1 antibody responses

(A) Representative IgG1 bacterial flow cytometric analysis of *A. muciniphila* incubated with the indicated mouse sera. Applies to results shown in C.

(B) Quantification of *A. muciniphila* colonization by fecal 16S qPCR for mice in (C).

(C) *A. muciniphila* IgG1 bacterial flow cytometric analysis for mice of the indicated genotypes and indicated *A. muciniphila* colonization status. WT *Akk*⁻ n=25, WT *Akk*⁺

n=41, *Tcrb*^{-/-} n=15. gMFI: geometric mean fluorescence intensity. Data are compiled from seven independent experiments.

(D) Quantification of *A. muciniphila* colonization by fecal 16S qPCR before (WT Akk⁻) and 5 weeks after (WT Akk-colonized) a single *A. muciniphila* oral gavage of 10⁹ cfu. n=6 mice. Applies to results shown in F. Data are representative of three independent experiments.

(E, F) Representative plot (E) and quantification (F) of *A. muciniphila* IgG1 bacterial flow cytometric analysis using sera from mice before (Akk⁻) and 5 weeks after colonization (Akk-colonized). n=5 mice. Data are representative of three independent experiments.

(G) Quantification of *A. muciniphila* colonization by fecal 16S qPCR. n=6 ASF, n=16 ASF + Akk mice. Data are representative of two independent experiments.

(H and I) *A. muciniphila* bacterial flow cytometric analysis with serial dilution of serum in ASF and ASF+Akk mice. Each line represents one mouse. The x-axis denotes total serum IgG1 (H) or serum IgA (I) concentration in the assay. n=9 mice per group. Data are representative of two independent experiments.

LoD: limit of detection. Each symbol represents a mouse, error bars represent mean ± SD. *p*-values were calculated with a Kruskal–Wallis test followed by Dunn’s multiple comparisons (B, C) or a Mann–Whitney test (D, F, G).

Fig. 3. *A. muciniphila* induces antigen-specific T follicular helper cell responses during homeostasis

(A) Representative flow cytometric analysis depicting transferred T cells (Thy1.1⁺) as percentage of all CD4⁺ T cells in the Peyer's patches of ASF and ASF+Akk mice 12 days after low-frequency adoptive transfer of Amuc124 TCR transgenic T cells. Quantified in (B).

(B) Frequencies of transferred T cells in intestinal tissues of ASF and ASF+Akk mice. n=5 mice per group, data are representative of six independent experiments.

(C and D) Representative flow cytometric analysis of expression of T follicular helper markers (PD-1, Bcl6, and CXCR5) by endogenous and transferred T cells in the Peyer's patches of ASF+Akk mice.

(E and F) Expression of T_H1 (T-bet⁺ FOXP3⁻), T_H2 (GATA3⁺ FOXP3⁻), T_H17 (RORγt⁺ FOXP3⁻), T_{reg} (FOXP3⁺), or T_{FH} (Bcl6⁺ PD-1⁺) markers by transferred T cells in the Peyer's patches (E) and small intestine lamina propria (F) of ASF+Akk mice. n=9 mice. Data are representative of six independent experiments.

(G) Representative Am3735-1 tetramer flow cytometric analysis of Peyer's patches from ASF and ASF+Akk mice.

(H) Frequencies of Am3735-1 or Am3740-1 tetramer⁺ endogenous T cells in ASF and ASF+Akk mice as a percentage of total CD4⁺ T cells. n=4 ASF, n=5-7 ASF+Akk mice. Data are representative of three (Am3740-1) or six (Am3735-1) independent experiments.

(I) Frequencies of Am3735-1 or Am3740-1 tetramer⁺ cells expressing T_{FH} markers (PD-1 and CXCR5, as shown in (J)) in the PP of ASF+Akk mice. n=5-7 mice, data are representative of three (Am3740-1) or six (Am3735-1) independent experiments.

(J) Representative flow cytometric analysis of expression of T_{FH} markers (PD-1 and CXCR5) by endogenous Am3735-1 or Am3740-1 tetramer⁺ cells.

(K) Numbers of Am3735-1 and Am3740-1 tetramer⁺ cells in all intestinal tissues in ASF and ASF+Akk mice. n=4 ASF, n=5 ASF+Akk mice. Data are representative of two (Am3740-1) or three (Am3740-1) independent experiments.

Each symbol represents a mouse, error bars represent mean ± SD. Gates on flow cytometry plots show mean±SEM. *p*-values were calculated with unpaired Student's *t*-tests (B, K), or a Mann-Whitney test (H)

Fig. 4. *A. muciniphila*-specific T cells also adopt other fates in the context of a complex microbiota

(A) Representative flow cytometric analysis depicting transferred T cells (Thy1.1⁺) as percentage of all CD4⁺ T cells in the Peyer's patches of SPF Akk⁻ and SPF Akk⁺ mice.

(B) Frequencies of transferred T cells as percentage of all CD4⁺ T cells in intestinal tissues of conventional specific pathogen-free (SPF) *A. muciniphila*⁻ (n=4) and SPF *A. muciniphila*⁺ (n=5) mice. Data are representative of three independent experiments.

(C and D) Expression of T_H1 (T-bet⁺ FOXP3⁻), T_H2 (GATA3⁺ FOXP3⁻), T_H17 (RORγt⁺ FOXP3⁻), T_{reg} (FOXP3⁺) or T_{FH} (Bcl6⁺ PD-1⁺) markers by transferred T cells in the Peyer's patches (C) or small intestine lamina propria (D) of SPF *A. muciniphila*⁺ mice. n=5 mice, data are representative of three independent experiments.

(E) Representative flow cytometric analysis of expression of T_H1 and T_H17 markers by endogenous total CD4⁺ T cells and *A. muciniphila*-specific (transferred) T cells in the SILP of SPF *A. muciniphila*⁺ mice.

Each symbol represents a mouse, error bars represent mean ± SD. Gates on flow cytometry plots show mean±SEM. *p*-values were calculated with unpaired Student's *t*-tests (B).