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B-Cell-Intrinsic MHCII Signaling Shapes Microbiota Composition

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Table of Contents

ABSTRACT

Recent studies demonstrate that immunoglobulin A (IgA), the most abundant antibody secreted at mucosal surfaces, is critical for limiting chronic inflammation but the mechanism by which this occurs is undefined. Regulation of the composition of the commensal bacterial community in the gut (i.e. the microbiota) could be one mechanism. IgA can be produced through T-cell-dependent (TD) and T-cell-independent (TiD) pathways. While TiD IgA is the most abundant IgA secreted into the gut, the relative contribution of TD and TiD IgA in regulating microbiota composition is controversial. Antigen presentation by B cells to T cells is essential for TD responses, and this is carried out by MHC class II molecules. Here, we sought to address the hypothesis that B-cell-intrinsic MHCII antigen presentation promotes anti-commensal TD IgA responses that influence microbiota composition. In order to do this, $RAG1^{-/-}$ mice, that lack their own T and B cells, received adoptive transfers of wildtype (WT) T cells along with MHCII-positive (MHCII+) B cells, or WT T cells along with MHCII-deficient (MHCII-) B cells. Results of our experiments demonstrate that the presence of MHCII on B cells leads to higher levels of IgA secretion into the gut, is critical to the formation of germinal centers, enhances binding of gut bacteria by high-affinity IgA, and is associated with greater species richness in the gut microbial community. Our results support that TD IgA responses promote species diversity in the gut, which is thought to benefit host health.

INTRODUCTION

Since Kircher's discovery of microorganisms in 1646, scientists have been curious about their biological relationships with humans [1]. Kircher speculated that agents he observed via microscopy in the blood of sick individuals were living organisms which could cause disease. Over the next several centuries, this concept, known as germ theory, gained significant support in the scientific community through the works of physicians like Semmelweis and Pasteur, who conducted experiments to describe the behaviors of the "animalicules" discovered by their predecessors and to connect them to infectious disease [2, 3]. By the late nineteenth century, microorganisms were generally accepted to be the causative agents of infection. However, scientists also observed that healthy individuals harbor microorganisms as well – for example, Koch discovered that some humans carry *Vibrio cholerae*, the bacteria responsible for cholera, and are entirely asymptomatic of the disease [4]. Metchnikoff was perhaps the first to describe the positive effects of what modern scientists call the microbiota – the community of microbes which reside on environmentally exposed surfaces of an animal. He wrote that there are "many useful microbes" and recommended the consumption of sour milk products cultured with *Bacillus bulgaricus* [5]. He believed the bacteria would propagate in an individual's gut and prolong one's lifespan through its secretion of lactic acid, which he believed could modulate the composition of the gut microbiota. Indeed, scientists have since confirmed that the composition of the human gut microbiota is vitally important to health and wellness and governed by a variety of factors.

The makeup of the microbiome has been shown to be responsive to variables like sex, age, diet, exercise levels, infection, and pharmacological treatment. Healthy gut microbiota are frequently characterized by high levels of diversity in species, and lack of diversity is correlated with dysbiosis – pathogenic effects caused by alterations to microbiota composition and/or function [6]. Gut dysbiosis has now been linked to numerous human diseases. Recent studies have revealed that gut dysbiosis is correlated not only with conditions that effect the bowels, like Crohn's disease and ulcerative colitis, but also with obesity, psychiatric disorders like Alzheimer's

and schizophrenia, liver disease, and a wide variety of others [7]. In light of these discoveries, it has become increasingly clear that it is important to elucidate the mechanisms through which the microbiome is regulated.

The immune system plays a crucial role in maintaining homeostasis and promoting diversity in the mammalian gut [8]. Because of the unique qualities of the gut environment, the mucosal immune system, which maintains gut homeostasis, is also unique. The intestine is filled with a plethora of harmless antigens from food and commensal bacteria, but also can contain pathogenic bacteria. In order to mediate this environment, the intestinal lumen is shielded from the lamina propria and deeper host tissues by a layer of mucous and epithelial cells [9]. One particularly important aspect of the interactions between the immune system and the bacteria of the gut is the secretion of antibodies into the intestine by terminally differentiated B cells (i.e. plasma cells). Antibody production in the intestine is stimulated through the uptake of antigens through the epithelial cells of the gut, most specifically through microfold cells (i.e. M cells) [10]. These cells selectively endocytose antigens and transport them to the basal side of the epithelium, presenting them to dendritic cells. These antigens are transferred to antigen-presenting dendritic cells, which transfer them to B cells in Peyer's patches and other gut-associated lymphoid tissues, who receive them via the B cell receptor. Subsequently, B cells endocytose these antigens, process them, and present them on their surfaces via major histocompatibility class II (MHCII) molecules. These cells subsequently move to the lymphoid follicles, where antigens are then presented to T follicular helper cells (T_{FH}), who, in tandem with B cells, form a germinal center and stimulate the proliferation of antibody-producing plasma cells which subsequently secrete antibodies [10].

Immunoglobulin A (IgA) is the most abundantly secreted antibody on mucosal surfaces and plays an important role in maintaining healthy bacterial diversity in the gut. It has been shown to coat up to 75% of all bacteria in the gut [11]. Despite being the most abundant immunoglobulin, IgA had long been considered to be an unimportant aspect of the adaptive immune response.

This belief was largely held due to the relatively mild phenotypes previously associated with selective IgA deficiency in humans. In recent times, we have learned that IgA can be critical to host health. IgA deficiency is the most common primary (i.e. heritable) antibody deficiency, and the presentation of this condition is highly variable between individuals. Clinically, IgA deficiency can go unnoticed, as screening for this antibody is not common in practice. However, studies which follow IgA-deficient patients longitudinally demonstrate that they are commonly affected by related ailments. Frequently, IgA-deficient subjects develop infections and suffer from food allergies. Less frequently, patients can develop asthma, skin infections, and even autoimmune diseases [12]. IgA-deficiency and food allergy are frequently linked together in literature, which leads scientists to question which mechanisms cause this connection to occur [13]. Food allergies, like many of the pathologies associated with antibody deficiency have been associated with gut dysbiosis and subsequent inflammatory responses. In order to understand this connection, we need to consider the mechanisms through which IgA is able to promote homeostasis and provide protection from pathogenic bacteria and potentially harmful antigens.

IgA is secreted in a dimerized form and can attach to bacterial cells via both its binding domains and through glycosylated side chains [14]. Generally, IgA binding mechanisms in the gut can be categorized into two types of activities – those that encourage commensal bacteria to proliferate, and those that protect the host from antigens and pathogenic bacteria. Immune exclusion is an important mechanism by which IgA protects host tissues. Immune exclusion is a process whereby antibodies physically block the interaction between microbes (or their harmful products) and host tissues. For example, it has been shown in mice that nonspecific IgA binding is sufficient to prevent the attachment of cholera toxin to the epithelium of the gut which protects animals from disease [15]. Toxins are then trapped in the mucosa or excreted and cannot cause harm to the intestinal barrier. This is also the mechanism frequently implicated in this antibodies' protection of the host against food allergens – through binding to these substances, the antibody can prevent them from entering the lamina propria, where they might encourage an inflammatory

response [13]. Additionally, it has been shown that bacterial cells coated with IgA are susceptible to agglutination, where these cells clump together in mechanisms spurred on by antibody binding and lose their propensity to bind to the cellular membranes of the gut and are also far less motile. One important aspect of this process is entrapment of bacteria in the mucosal lining which coats the apical side of the intestinal epithelium. Because IgA is heavily glycosylated, it concentrates in the mucus further aiding in entrapment of bacteria in the mucus. Entrapment prevents pathogenic microorganisms from transcending the mucosa and colonizing the submucosa. Interestingly, pathogenic bacteria have been shown to be much less prone to replication after IgA attachment, showing that the antibody can root out potential challenges to the homeostasis of the microbiota composition by quenching virulence. For example, in studies done with *Shigella flexneri*, IgA binding to the O antigen (a major virulence factor) prevented the bacteria from binding to epithelial cells and decreased membrane potential, indicating that IgA can directly inhibit bacterial virulence [16]. Additionally, secretory IgA is capable of limiting the pro-inflammatory effects of luminal microbes when they do colonize the mucus lining. For example, in rabbit models, it was demonstrated that IgA has a vital role in preventing *S. flexneri* from upregulating inflammatory genes in immune cells of the Peyer's patches (lymphoid tissues embedded in the wall of the small intestines of mammals) [17].

In addition to its protective properties, IgA promotes the proliferation of beneficial bacteria and encourages diversity. *Bacteroides fragilis*, a commensal bacterium in the gut, is demonstrated to utilize IgA binding in order to adhere to the mucosal epithelium in the gut, which is a niche in which it thrives. In this instance, IgA binding to carbohydrates on the cellular surface of *B. fragilis* encourages the antibody-coated bacteria to associate closely with the epithelial cells and mucous membranes of the intestine, and aggregate in this ecosystem. Donaldson et al. demonstrated that in $IgA^{-/-}$ mice, the *Bacteroides* family was unable to colonize the lamina propria [18]. In mice, it has been shown that the composition of the microbiota changes from a proinflammatory "immature" to anti-inflammatory "mature" state over time. Using a $RAG^{-/-}$ model, it was

demonstrated that mice lacking an IgA targeted response against γ-proteobacteria, specifically *Enterbacteriaceae*, had high levels of colonic inflammation as compared to IgA-producing mice [19]. IgA-producing wild-type mice had higher levels of bacterial diversity in the microbiota and the community was not dominated by proinflammatory species, in contrast to the experimental group that lacked IgA production. This demonstrates the vital role of IgA in promoting the diversity of the microbiome through suppression of harmful bacteria, thereby giving helpful commensals an opportunity to proliferate. Additionally, it has recently been suggested that IgA binding can induce direct regulatory effects on genes in commensal bacteria. In studies done focusing on the model commensal bacteria *Bacteroides thetaiotamicron*, it has been shown that IgA binding upregulates the production of MAFF (mucus-associated functional factor). The downstream effects of this upregulation are intriguing; production of MAFF by *B. thetaiotamicron* results in larger bacterial cells and increases their growth rate. *B.thetaiotamicron*-produced MAFF also promotes the growth of groups of bacteria within the genus *Clostridium*. Interestingly, the upregulation of MAFF also increases epithelial cell proliferation in the intestine, which enhances gut barrier function and further protects the host [20].

MHCII molecules are of particular interest due to their importance in shaping adaptive immune responses and the fact that they are some of the most genetically polymorphic genes found in vertebrates. MHCII molecules are glycoproteins that bind protein (i.e. peptide) antigens and present them on the cell surface where they can stimulate T cell activation. B cells express MHCII, and the interaction with T cells is central to the development of TD IgA response [21]. In the TD pathway of B-cell activation (the focus of this paper) antigen-presenting cells (APCs), which include B cells, utilize MHCII to present antigens to T cells. These MHCII:peptide complexes stimulate T cells via their T cell receptor (TCR). This causes T cell activation and subsequent crosstalk between T cells and B cells that ultimately results in the maturation of B cells into plasma cells that secrete antibodies. The importance of this response as compared to the TiD pathway of B cell activation lies in the specificity of antibodies produced through each

pathway. In several ways, it has been demonstrated that specific IgA responses do have a role in modulating the microbiota. For example, Kawamoto et al. demonstrated that T-cell transfer into T-cell deficient mice, thereby allowing for T_{FH} cell proliferation and TD B cell activation, was associated with enhanced species diversity within gut microbial communities [22]. Several papers also describe the importance of MHCII on group 3 innate lymphoid cells (ILC3) in controlling the composition of the gut microbiota via regulation of $CD4^+$ T cell activation and the magnitude of the ensuing IgA response. These cells are innate immune cells which reside within the gut lamina propria as well as gut-associated lymphoid tissues (e.g. mesenteric lymph nodes). Hepworth et al. showed that in mice with deletions of MHCII on ILC3, the innate immune response to commensal bacteria was inadequately regulated and therefore resulted in proinflammatory $CD4^+$ T cell responses proving that MHCII is the mechanism through which these cells interact [23]. ILC3s serve to downregulate TD B cell activation through MHCII-dependent interactions with $CD4^+$ T cells, seeming to provide some regulation between adaptive and innate immune responses against commensals in order to maintain homeostasis [24]. MHCII has also been shown to be influential in controlling gut microbiota when expressed on dendritic cells, as it is vital for the presentation of antigens processed from commensal bacteria, which in turn drivers T_H17 cell differentiation [25].

The literature clearly demonstrates that MHCII is influential in regulating the composition of the gut microbiota. However, very little is known about the importance of MHCII expression on B cells in regulating microbiota composition and maintaining gut homeostasis. Here, using a $RAG1^{-/-}$ adoptive transfer model, we sought to address the hypothesis that B-cell-intrinsic MHCII antigen presentation significantly affects microbiota diversity. Results from our experiments demonstrate that, as expected, B-cell-intrinsic MHCII is required for germinal center formation in gut Peyer's patches, that B-cell-intrinsic MHCII facilitates the generation of high affinity anti-commensal IgA responses, and that this is associated with a significant increase in microbiota species richness.

METHODS

Mouse models. A long-term breeding colony of WT, RAG1$^{-/-}$, and MHCII$^{-/-}$ mice (all C57BL/6 background) has been maintained by the Kubinak Lab at the University of South Carolina for three years. WT (Jax#000664), RAG1$^{-/-}$ (Jax#002216), and MHCII$^{-/-}$ (Jax#003584) mice were originally purchased from Jackson laboratories. All animals used in the experiments described here were derived from this colony. Male and female mice were used in all experiments. Mice were reared and maintained in a single environmentally controlled room exclusively used to house this mouse colony. Mice were maintained under constant environmental conditions (70^{o}F, 50% relative humidity, 12:12 light:dark cycles) and were given *ad libitum* access to autoclaved drinking water and an irradiated soy-free mouse chow (Envigo; diet#2920X). All animal use strictly adhered to federal regulations and guidelines set forth by the University of South Carolina Institutional Animal Care and Use Committee (Protocol#101292).

Flow Cytometry. Flow cytometry was performed on a BD Aria II cell sorter or BD Accuri C6 cytometer. For cell staining, 500,000 cells per animal were stained with appropriate antibody cocktails. All antibodies were used at a final concentration of 1:250 with the exception of MHCII, which was stained at 1:500 concentration. Cells were stained in 100 μL volumes in the dark for 20 minutes. Stained cells were then washed twice with 1X wash buffer. Washed cells were fixed in 2% paraformaldehyde for 10 minutes and then analyzed on instrument. Purity of B and T cell isolations were determined by staining the spleen cells (after isolation) with CD4 and B220 antibodies. Peyer's Patches (PPs) were collected from the small intestines of mice, crushed and stained. There were two panels done on the PPs. Germinal center (GC) B cells and GC-T follicular helper cells (T_{FH}). GC-B cells were identified as B220^{+}IgD^{lo}Fas^{+}GL7^{+}. GC-T_{FH} cells were identified as CD4^{+}B220^{-}CXCR5^{hi}PD1^{hi}.

Adoptive B Cell Transfers. RAG1$^{-/-}$ mice were randomly assigned to treatment groups for B cell transfer experiments. Treatment groups received 1.0x10^{7} B cells isolated from the spleens of sex and age-matched WT or MHCII$^{-/-}$ donors via magnetic purification using the EasySep Mouse CD19 Positive Selection Kit II (STEMCELL: catalog # 18954). This kit obtains a cell purity of 98%. The treatment groups also received 1.0x10^{5} T cells isolated from the spleens of the WT donors via magnetic purification using the EasySep Mouse Naïve CD4^{+} T Cell Isolation Kit (STEMCELL: catalog #19765A). This kit obtains a cell purity of 98%. On day 0, B cells and T cells were administered via intraperitoneal injection (in 200uL volumes) and then animals were singly housed for ten weeks. At ten weeks post transfer, animals were sacrificed, and tissues were collected for analysis. For the second replicate, B cells and T cells were administered via intraperitoneal injection (in 200uL volumes) on day 0, and then animals were singly housed for seven weeks. At seven weeks post transfer, animals were sacrificed, and tissues were collected for analysis. Detection of fecal IgA was used as confirmation of successful B cell engraftment. A control group was done by administering isolated (age-matched) MHCII+ B or MHCII- B cells into RAG1$^{-/-}$ mice. The animals were singly housed for seven weeks after the B cells were administered via intraperitoneal injection. At seven weeks post transfer, animals were sacrificed, and tissues were collected for analysis. Based on these criteria, we determined that i.p. injection of donor B cells resulted in detectable fecal IgA in 100% of RAG1$^{-/-}$ recipient mice.

Measurement of Fecal IgA. Longitudinal sampling of fecal pellets from experimental mice was performed to quantify the dynamics of IgA responses across adoptive transfer treatment groups. To do this, fecal pellets were collected immediately prior to adoptive transfer and then at weekly intervals. Fecal pellets were crushed in 500μL of Hank's Balanced Salt Solution and centrifuged at 4000x RCF for 10 minutes. Subsequently, the supernatant was transferred to new tubes and centrifuged at 8000 x RCF for 10 minutes. The remaining supernatant was collected. These samples were subsequently measured for IgA content by ELISA, using the Invitrogen IgA Mouse

Uncoated ELISA kit (catalog # 88-50450-88). The IgA content of these samples was determined, and the values for the groups of mice given MHCII+ and MHCII- B cells were corrected for noise by subtracting the standardized IgA concentration values generated from the control group samples.

Measurement of IgA-bound bacteria. Pellets were collected from mice at the end of the experiment. Pellets were crushed and resuspended in 1 mL of 1X PBS and refrigerated for 20 minutes. The samples were then homogenized and centrifuged. Supernatant was passed through a 100μm strainer and centrifuged again. The pellets were resuspended in 1 mL of column buffer and centrifuged. The pellets were resuspended in 200μL of column buffer and 4μL of IgA antibody and 4μL of Ig kappa light chain antibody were added to each sample. The stained samples were then covered and refrigerated for 30 minutes. The samples were then centrifuged and washed 2X with 1XHBSS (without salts). The pellets were then resuspended in 200μL of 1XHBSS and 10μL of 1X SYBR green was added to each sample. The samples were then covered and incubated at room temperature for 5 minutes. Fully stained samples were added to 2% PFA and 1 mL of 1XHBSS.

16S microbiota profiling. Fecal samples and small intestinal contents were collected from mice for 16S rRNA gene sequencing. For fecal samples, animals were scruffed and 1-2 fecal pellets were directly sampled from mice by having them defecate directly into a 1.5mL microfuge tube. All samples were frozen at -80^{o}C until DNA extractions were performed. DNA was extracted using the QIAamp Powerfecal Pro DNA Isolation Kit (Qiagen) with 3 minute bead-beating step. Purified DNA was sent to University of Alabama Birmingham Heflin Center Genomics Core for 16S sequencing on an Illumina MiSeq. Raw fastq reads were de-multiplexed and forward and reverse primer sequences were trimmed from reads. This yielded a 251bp product spanning the V3/V4

region of the bacterial 16S rRNA gene. All 16S analyses were carried out using QIIME 2.0 analysis pipeline.

Statistical Analysis.

Data sets for all experiments represent pooled data from two to three experimental replicates (n=2-3 animals per replicate). For adoptive transfer experiments, animals were treated and then individually housed for the duration of experiments. Thus, each animal represents an independent replicate. Unless otherwise noted in figure captions, a Student’s t-test was used for significance testing with a Welch's correction applied for comparison between data sets with unequal variance. All univariate statistical analyses were performed using PRISM8.0 Statistical Analysis Software (Graphpad).

RESULTS

Adoptive transfer of WT B cells leads to higher levels of IgA production

In order to test our hypothesis, transgenic RAG1$^{-/-}$ mice were randomly segregated into five experimental groups; four that were modified via adoptive transfer of immune cells and one control group that was given an injection of saline. The remaining four groups received the following adoptive transfer treatments; WT B cells only, MHCII- B cells only, WT T cells and MHCII+ B cells, or WT T cells and MHCII- B cells (Figure 1A). These mice had weekly ELISA quantification of fecal IgA performed, which yielded insights into the relative IgA concentration changes caused by MHCII presence on B cells and the role of T cells in generating antibodies. Mice who received WT B cells only (hatched blue line) produced relatively more IgA as compared to those which received MHCII- B cells only (hatched red line)(Figure 1B). The presence of T cells (solid blue line) did not cause significantly more IgA production relative to mice receiving only MHCII+ or MHCII- B cells (figure 1B).

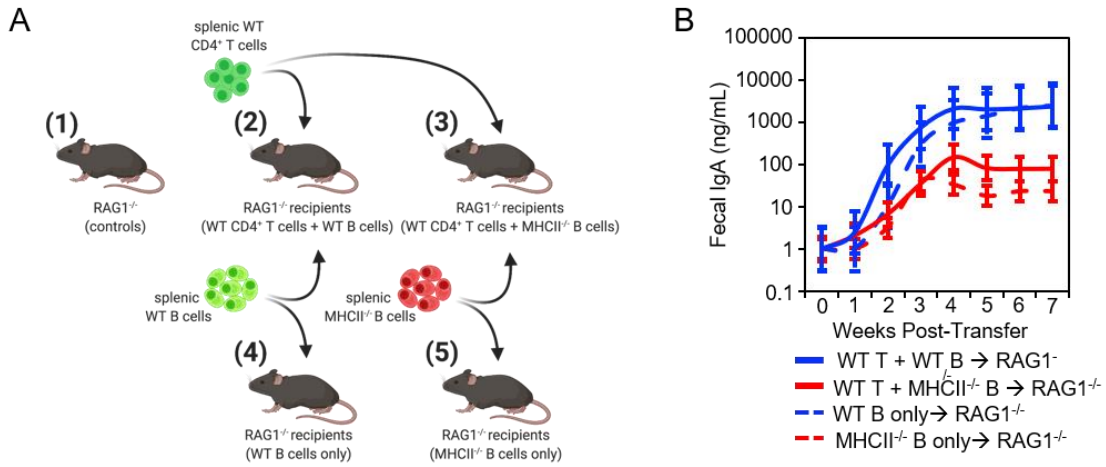

Figure 1. Study design and IgA responses in RAG1$^{-/-}$ adoptive cell transfer recipient mice. **(A)** Five experimental cohorts of mice were used in this study and include the following groups: (1) RAG1$^{-/-}$ control mice that did not receive cells, (2) RAG1$^{-/-}$ mice that received splenic WT CD4^{+} T cells and splenic WT B cells, (3) RAG1$^{-/-}$ mice that received splenic WT CD4^{+} T cells and splenic MHCII$^{-/-}$ B cells, (4) RAG1$^{-/-}$ mice that received splenic WT B cells only, and (5) RAG1$^{-/-}$ mice that received splenic MHCII$^{-/-}$ B cells only. **(B)** Fecal IgA concentrations are shown for seven weeks post-cell transfer for each experimental cohort of RAG1-/- recipients (i.e. cohorts 2,3,4,5 shown in A).

B-cell-intrinsic MHCII coordinates germinal center activity

Flow cytometry gating of Peyer's-patch-derived immune cells from control and experimental groups provided qualitative and quantitative evidence to suggest that B-cell intrinsic MHCII activity is crucial for the formation of germinal centers. Events were grouped into two primary groups: $B220^{-}CD4^{+}$, indicative of $CD4^{+}$ T cells, and $B220^{hi}IgD^{+}$, indicative of B cells. T cell events were then gated by PD1 and CXCR5 markers – $PD1^{lo}CXCR5^{lo}$ events were classified as T_{FH} cells; $PD1^{hi}CXCR5^{hi}$ events were classified as germinal center T_{FH} cells (GC-T_{FH}). B cell events were segregated by $B220^{hi}IgD^{hi}$, categorized as naïve B cells, and $B220^{+}IgD^{lo}$ events being categorized as activated B cells. Of the activated B cell population, $FAS^{+}GL7^{+}$ events were considered as germinal center B cells (figure 2A). The data indicates that as compared to mice which received MHCII- B cell and WT T cell adoptive transfers, those which received MHCII+ B cells and WT T cells maintained similar percentages of $CD4^{+}$ T cells in Peyer's patches, as compared to total lymphocytes. However, the group with WT B cells had significantly higher proportions of T_{FH} cells and much higher proportions of GC-T_{FH} cells (Figure 2B). Additionally, mice with WT B cells and WT T cells had significantly higher proportions of B cells as compared to all lymphocytes, higher proportions of activated B cells, and higher proportions of germinal center B cells as a proportion of all activated B cells (figure 2B).

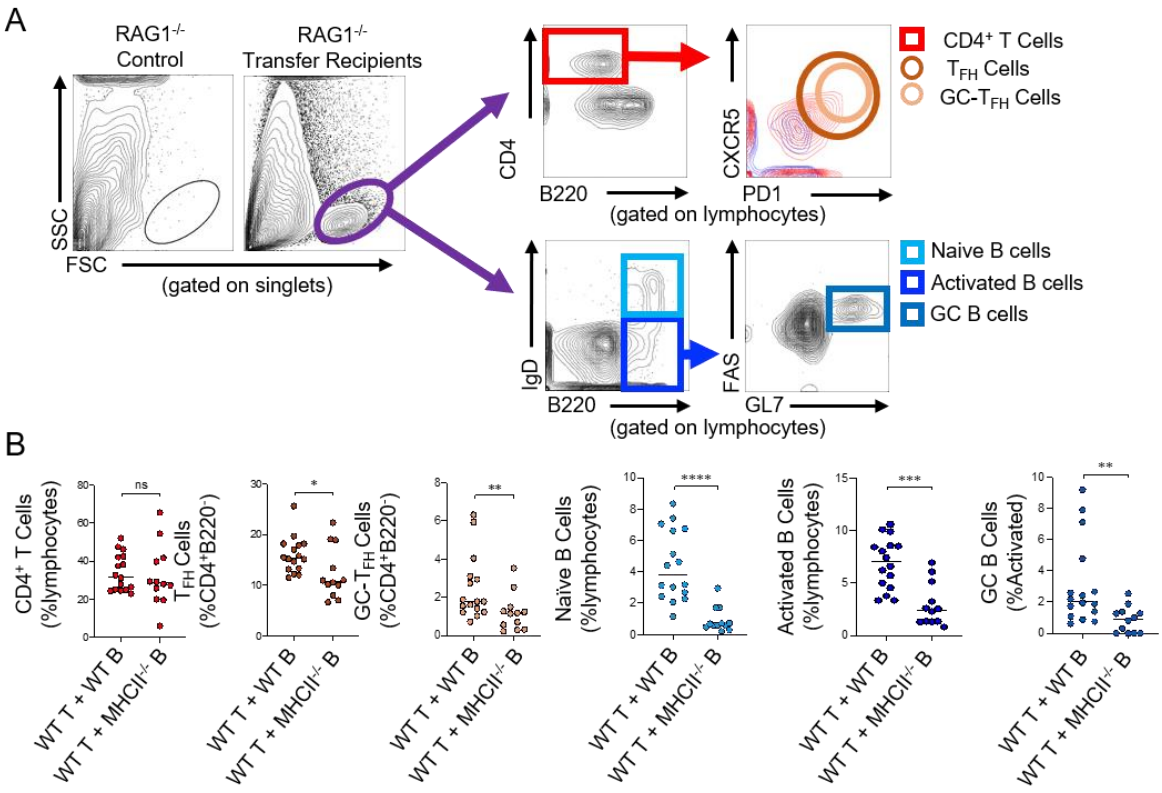

Figure 2. B-cell-intrinsic MHCII is required for GC responses to develop in the PPs of RAG1-/- transfer recipients. (A) Representative gating strategy for enumeration of T cell and B cell subsets in cell transfer recipient RAG1$^{-/-}$ mice. (B) The relative abundance of relevant T and B cell subsets in PPs is shown. Student's t-test; ns=not significant, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

B cell MHCII increases IgA-bacteria binding specificity

Fecal samples were collected and processed for flow cytometry to quantify the presence and abundance of IgA-bound bacteria. SYBR+ events were considered to represent bacteria, and these events were in turn gated on for IgA and Igκ positivity. SYBR^{+}IgA^{+}Igκ^{+} events were recognized as IgA-bound bacteria (figure 3A). The data shows that RAG1$^{-/-}$ mice receiving only MHCII+ B cells contain significantly higher percentages of IgA-coated bacteria within their feces as compared to RAG1$^{-/-}$ mice receiving only MHCII- B cells, and those RAG1$^{-/-}$ mice receiving WT T and MHCII+ B cells contained an even higher percentages of IgA-bound bacteria as compared to the MHCII+ B cell only group Figure 3B).

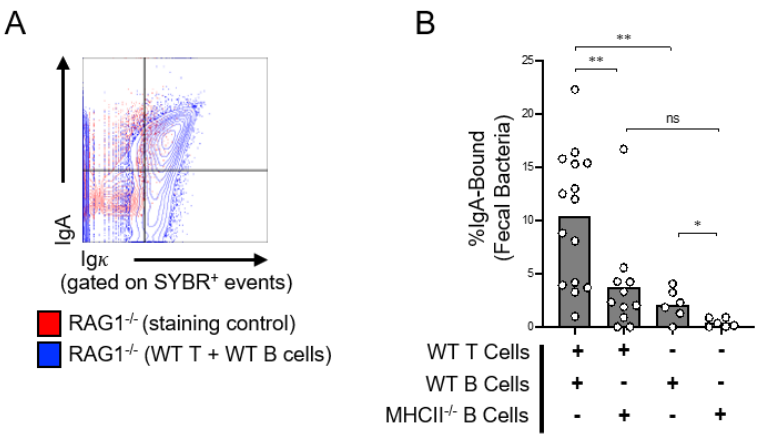

Figure 3. B-cell-intrinsic MHCII is required for high-affinity anti-commensal IgA responses in the SI. (A) Representative plot demonstrating gating strategy used to enumerate IgA-bound bacteria in the feces of RAG$^{-/-}$ transfer recipients. **(B)** Results of assay are shown. Student's t-test; ns=not significant, *=p<0.05, **=p<0.01.

B-cell-mediated mechanisms influence microbiota species diversity

Microbiota composition was characterized by sequencing the bacterial 16S rRNA gene, and sequencing data was analyzed using the QIIME2.0 analysis pipeline. Unweighted UniFrac analysis demonstrated that between the experimental groups receiving WT T cells and either MHCII+ B or MHCII- B cells, no significant differences in phylogenetic diversity were observed (figure 4A). However, when the total number of species observed within these communities are compared, we observed that RAG1$^{-/-}$ mice receiving WT T and MHCI+ B cells maintained a significantly more species-rich microbial community as compared to RAG1$^{-/-}$ control mice (no cells) and RAG1$^{-/-}$ mice receiving WT T cells and MHCII- B cells (Figure 4B). Interestingly, RAG1$^{-/-}$ mice with no adoptive transfer demonstrated higher levels of species diversity as compared to mice with adoptively transferred WT T cells and MHCII- B cells (figure 3B).

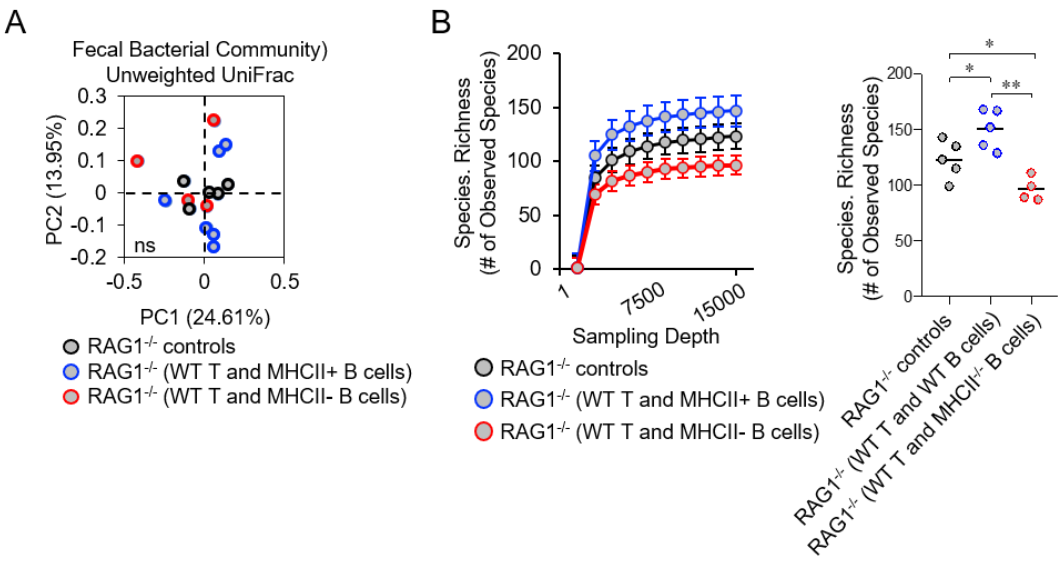

Figure 4. B-cell-intrinsic MHCII increases microbiota species richness. (A) A PcoA plot comparing β–diversity estimates (unweighted UNIFRAC) among fecal bacterial communities is shown. PERMANOVA; ns=not significant. **(B)** An α-rarefaction plot is shown demonstrating equal sampling of species diversity among experimental cohorts. Statistical comparison of species richness estimates among are shown. Student's t-test; *=p<0.05, **=p<0.01.

DISCUSSION

The results of this study show that the presence of MHCII on B cells directs IgA-based responses which shape the composition of the microbiota. Through adoptive transfer models, quantitative evidence indicating that MHCII presence on B cells generates a significantly higher level of IgA secreted into the gut as compared to mice receiving MHC- B cells was generated. Additionally, the crucial role of B-cell-intrinsic MHCII in the promotion of germinal center formation in gut Peyer's patches and the resulting generation of high-affinity anti-commensal IgA responses was confirmed. MHCII-facilitated IgA responses to commensal microbiota were shown to be of higher affinity as compared to those induced through alternative pathways, and quantitative differences in this response were shown to be associated with levels of species diversity in the feces.

The data generated through ELISA quantifications of fecal IgA concentration clearly indicate the influential nature of B-cell-intrinsic MHCII in mucosal antibody secretion. Snider et. al previously reported that general MHCII ablation in the C2D strain of mice resulted in similar levels

of total IgA as measured by ELISA, suggesting that the presence of MHCII does not cause a change in total IgA production in feces, except in the situation of an immune response to a protein antigen[26]. While our study focuses on the effects of B-cell intrinsic MHCII on IgA production, MHCII presentation on other lymphocyte populations influences levels of IgA in the gut as well (e.g. dendritic cells and type 3 innate lymphoid cells) [27]. MHCII on dendritic cells is capable of presenting antigens to T cells, promoting their differentiation into regulatory T cells and T_H17 cells, which have subsequent downstream effects on antibody production [28]. MHCII presentation on ILC3 is capable of stifling antibody-generating interactions between T_{FH} cells and B cells; ablation of MHCII from ILC3s is associated with increased levels of IgA secretion [24]. MHCII on cells other than B cells is relevant to total IgA levels in the body, and results from the C2D model suggests that there are compensatory IgA-generating responses induced when MHCII is absent. Interestingly, the whole-body MHCII knockout model provides much context for the results observed in this experiment. By comparing IgA concentrations in the experimental groups which received MHCII+B cells and WT T cells, and those which have MHCII+ B cells and no T cells, we can affirm that TD antibody generation pathways do not generate a large fraction of the IgA secreted into the gut. However, we also observe that MHCII+ B cells do generate a significant amount of IgA in the gut relative to MHC- B cells (figure 1B). In some manner, MHCII on B cells is clearly relevant for IgA generation through TiD pathways; the mechanisms behind this phenomenon have not been described in the literature to our knowledge, and investigating these mechanisms is an excellent avenue for future inquiry. One interesting possibility is that B-cell-intrinsic MHCII signaling may allow B cells to interact with an as-of-yet undefined $CD4^+$ T cell subset that is able to develop in $RAG1^{-/-}$ mice.

The germinal center (GC) of the Peyer's patch is a crucial micro-environment for high affinity immunoglobulin production in the intestine, and MHCII is highly influential in the signaling which influences the actions cells take within these environments [29]. Dendritic cell presentation of antigen to T cells is mediated through MHCII, and this interaction is crucial for maintaining

specificity in immune responses through differentiation of $CD4^+$ T cells into regulatory T cells and T_H17 cells [30]. These cells have been shown to regulate T_{FH} secretion of IL-22 in the Peyer's patch, subsequently leading to increased concentrations of IgA-producing plasma cells [31]. MHCII presentation of antigen on B cells is most crucial in governing the environment of the Peyer's patch as well. Our data indicates that whilst MHCII presence on B cells does not drastically influence the percentage of $CD4^+$ T cells in the Peyer's patch, it certainly does influence the composition of these lymphocytes; a significantly higher percentage of T_{FH} cells of total T cells is present in mice with WT B cells as compared to those without, and this trend is even more dramatically emphasized by the percentage of GC-T_{FH} cells (figure 2B). This data affirms that MHCII+ B cells activate T cells to induce a germinal center response. Gating of the Peyer's patch for B cell markers indicated that there was a higher percentage of B cells as a function of total lymphocytes in the population in MHCII+ B cell and WT T cell transfer groups, and a higher percentage of activated and GC-associated B cells. The relative abundance of B cells in the experimental group is likely caused by the formation of germinal centers, and the clonal proliferation of activated B cells forming in the germinal center reaction. The higher levels of activated B cells is also intrinsically related to MHCII presentation; B cell activation of T cells through MHCII-TCR interactions in turn leads to T cell secretion of factors which encourage the proliferation of the B cells into IgA producing plasma cells.

Whilst MHCII presence on B cells does increase the quantity of IgA secreted in the experimental groups of mice in this experiment, it is most importantly related to the quality, i.e. affinity, of the IgA secreted into the intestine. Flow cytometry of fecal bacteria determined that the highest percentages of IgA-bound bacteria were found in the experimental group with both WT T and MHCII+ B cells, and that the WT T and MHC- B cell transfer group had less than half the quantity of IgA-bound bacteria (figure 3B). Given this difference, MHCII on B cells must be important for generating high-affinity antibody responses to bacterial antigens. However, the experimental group receiving MHCII+ B cells only demonstrated very low levels of IgA binding to

bacteria despite having comparable concentrations of IgA in their gut, indicating that the TD-antibody generation pathway is primarily responsible for elevating the quality of IgA secreted.

High-affinity binding of IgA to the microbiota has been correlated to increased species diversity and gut health [31]. Therefore, we sought to determine if this phenomenon is occurring in our model and performed 16S rRNA gene sequencing to answer this question. Whilst general phylogenetic composition was not observed to be have been altered between RAG1$^{-/-}$ mice receiving either MHCII+ B and WT T cells, MHC- B and WT T cells, or control mice (i.e. no cells), the total numbers of species in the gut were shown to have been drastically altered by treatment method (figure 4). The most species diversity was observed in the RAG1$^{-/-}$ mouse cohort receiving WT T and MHCII+ B adoptive transfer model, indicative of the promoting effect of high-affinity IgA binding to bacteria on general gut microbiota diversity. The least species diversity was, interestingly, observed in the RAG1$^{-/-}$ cohort receiving WT T and MHCII- B cells. The IgA these mice secreted bound to a smaller percentage of bacteria and was evidently much less selective. MHCII has previously been described to be regulatory of T cell differentiation and proliferation, and ablation of MHCII has been associated with hyper-inflammatory responses in the gut related to the hypersecretion of cytokines by CD8^{+} T cells [32]. Do et al. demonstrated that RAG1$^{-/-}$ mice into which CD8^{+} T cells from MHCII$^{-/-}$ mice were adoptively transferred, developed colitis. Previous studies have shown a strong correlation between decreased microbiota diversity and inflammatory bowel disease [33]. This information provides a clear framework on which to view our results; in our WT T and MHCII- B cell model, lack of regulation from B cells may be inducing hyperactive T cell responses which resulted in gut inflammation and subsequent dysregulation of the microbiota, which may be responsible for the decreased species number we observe in these animals.

The connection between our gut microbiome's composition and its effects on our health has been increasingly examined in the past decade. The composition of the microbiome has been shown to effect human disease, and vice versa. One of the primary measures through which

scientists have been able to quantify the quality of the microbiota to examine this phenomenon is through study of species diversity, frequently using 16S rRNA gene sequencing. With this tool, the number and phylogeny of bacterial species in a fecal sample can be determined. Species number and diversity have been positively correlated to good health outcomes. Frank et al. wrote of the connection of microbiota species diversity to common bowel diseases in humans, such as ulcerative colitis, and Crohn's disease. The authors found that in human subjects, these intestinal diseases were strongly correlated with decreased numbers of both total bacteria present in the gut and number of species present in the gut, and dysbiosis in terms of groups of bacteria such as *Bacillus* were overrepresented relative to the gut community of healthy individuals [34]. The composition of the microbiome is bi-directionally related to the immune processes which influence it; bacterial metabolic byproducts, like butyrate released by *Clostridia*, leads to regulatory T cell differentiation and induction of tolerant immune responses against the microbiota [35]. Specific bacterial species have also been linked with activation of immune responses; segmented filamentous bacteria, a species which grows in close association with the mucosa, has been shown to induce $CD4^+$ T cell differentiation into T_H17 cells, which in turn produce interleukins which regulate T cell activation [36]. A wide variety of bacteria function in tandem to regulate the composition of the microbiota and preserve homeostasis within the gut, thereby promoting host health. Therefore, upkeep of microbiota diversity is highly important.

Through promotion of germinal center activity, MHCII on B cells is highly important to regulating the quantity and quality of the IgA response to commensal bacterial in the gut. By allowing the formation of the germinal center, B cells promote high-affinity TD IgA responses in the gut. We have demonstrated that these said responses lead to high-affinity IgA binding of commensal bacteria, which can regulate the composition of the microbiota through various pathways, including controlling bacterial virulence and downstream effects on T cell activation. Thereby, we have determined that B-cell-intrinsic MHCII signaling leads to a more diverse microbiota, a characteristic associated with health and wellness in humans and mice alike. Our

adoptive transfer model both answers the question of the value of B-cell-intrinsic MHCII signaling in controlling the gut microbiota through TD IgA antibody secretion, but also suggests that B-cell-intrinsic MHCII may be playing a currently unappreciated role in T-cell-independent IgA production in the gut.

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AUTHOR CONTRIBUTIONS

J.L.K. designed and oversaw all experiments. M.M.R., S.A., and J.L.K. wrote the first and final draft of this manuscript. M.M.R. performed all experiments and data analysis. S.A. and N.H. performed longitudinal fecal sampling from mice, performed fecal ELISAs, assisted in flow cytometry, and edited draft revisions. A.J. maintained experimental mice and assisted in sample collection.

COMPETING INTERESTS

The authors have no competing interests to disclose.

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