



RESEARCH PAPER



Dynamic immunoglobulin responses to gut bacteria during inflammatory bowel disease

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ABSTRACT

Aberrant immune responses against gut microbiota are thought to be key drivers of inflammatory bowel disease (IBD) pathogenesis. However, the extent and targets of immunoglobulin (Ig) A versus IgG responses to gut bacteria in IBD and its association with IBD severity is not well understood. Here, we address this by analyzing fecal samples from Crohn's disease (CD), ulcerative colitis (UC), and Non-IBD patients by flow cytometry for the frequency of bacteria that were endogenously bound with IgA and/or IgG. Assessment of IBD patients from two geographically distinct cohorts revealed increased percentages of IgA- and IgG-bound fecal bacteria compared to non-IBD controls. Notably, the two major subsets of IBD showed distinct patterns of Ig-bound bacteria, with CD activity associated with increases in both IgA and IgG-bound bacteria, whereas UC activity correlated only with increases in IgG-bound bacteria. Analysis of the flow sorted Ig-bound bacterial repertoire by 16S rDNA sequencing revealed taxa that were Ig-bound specifically in IBD. Notably, this included bacteria that are also thought to reside in the oral pharynx, including *Gemella*, *Peptostreptococcus*, and *Streptococcus* species. These data show that the pattern of IgA and IgG binding to fecal bacteria is distinct in UC and CD. In addition, the frequency of Ig-bound fecal bacteria may have potential as a non-invasive biomarker for disease activity. Finally, our results support the hypothesis that immune responses to oral pharyngeal bacteria may play an important role in the pathogenesis of IBD.

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Introduction

Inappropriate immune responses against commensal bacteria are thought to contribute to the pathogenesis of inflammatory bowel disease (IBD).^{1,2} However, our understanding of the extent of the immune response to gut bacteria in IBD is incomplete.³ Immunoglobulin A (IgA) is abundantly secreted into the gastrointestinal tract and has been proposed to target gut bacteria.^{4–9} To evaluate if IgA targets specific bacteria *in situ*, we and others have combined flow-cytometric sorting with 16S rDNA sequencing.^{7,8} Using this approach, it was reported that IBD patients demonstrated increased targeting of fecal bacteria by host IgA.^{5,7} and IgG.⁵ with a potential difference in active and inactive IBD patients.⁵ In addition, IgA-tagged microbes from human patients with IBD or malnutrition were more pathogenic than non-tagged

microbes when transferred into germ-free mice.^{7,8} Thus, the specificity of IgA may be useful to identify the bacterial taxa that elicit adaptive immune responses and contribute to disease pathogenesis.

However, a number of questions remain regarding the Ig-targeting of commensal bacteria in IBD. First, there are major differences in the disease pathology in IBD subsets, Crohn's disease (CD) and Ulcerative colitis (UC), but it remains unclear whether they exhibit differences in their pattern of IgA and IgG-binding to fecal bacteria. Second, IBD is a relapsing and remitting inflammatory disorder, yet it remains unknown whether the IgA- or IgG-bound bacteria vary with disease activity. Last, IgG-targeted bacteria represent a large proportion of the total immunoglobulin responses against bacteria in IBD patients, but the bacterial taxa bound by IgG in the gut remain unknown.

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We addressed these questions using bacterial FACS on IBD fecal specimens from two different patient cohorts. Our data also reveal distinct differences between CD and UC in the percentage of IgA- and IgG-bound bacteria with disease activity, as well as the taxa of bacteria targeted by Ig. Although future studies are required, these data suggest that flow cytometry of fecal Ig-bound bacteria may provide insight into the bacteria involved in IBD pathogenesis and may have potential as a useful biomarker for the assessment of IBD disease activity.

Results

IBD patients demonstrate increased ig-bound bacteria

To investigate whether IBD subjects display exaggerated Ig responses against commensal bacteria surface antigens, we analyzed using flow cytometry (Figure 1A) frozen fecal specimens from 30 CD, 14

UC and 30 Non-IBD subjects from St. Louis (STL) (Table 1). This study was powered to detect a 0.5 effect size in the frequency of Ig binding (ANOVA 3 groups) based on previously reported results for IgA in IBD,⁷ which had a smaller sample size for CD and UC patients. We also analyzed 3 CD, 11 UC and 9 Non-IBD subjects from Cambridge (CBG, Table 1) to provide independent, albeit limited, supplemental data. The technique was reproducible, as flow cytometry performed on the same sample on different days did not show significant difference (Fig. S1A). In both cohorts, UC and CD subjects showed significantly higher frequencies of IgA-bound fecal bacteria compared with Non-IBD subjects (Figure 1B, Fig. S1B), consistent with previous studies.^{5,7} IgG-bound fecal bacteria were detected only in UC and CD, but not Non-IBD, subjects (Figure 1B, S1C). Although Ig-binding was still enhanced in the IBD vs Non-IBD patients in the CBG cohort, the range of Ig+ bacteria was lower as compared to STL, possibly due to geographic or

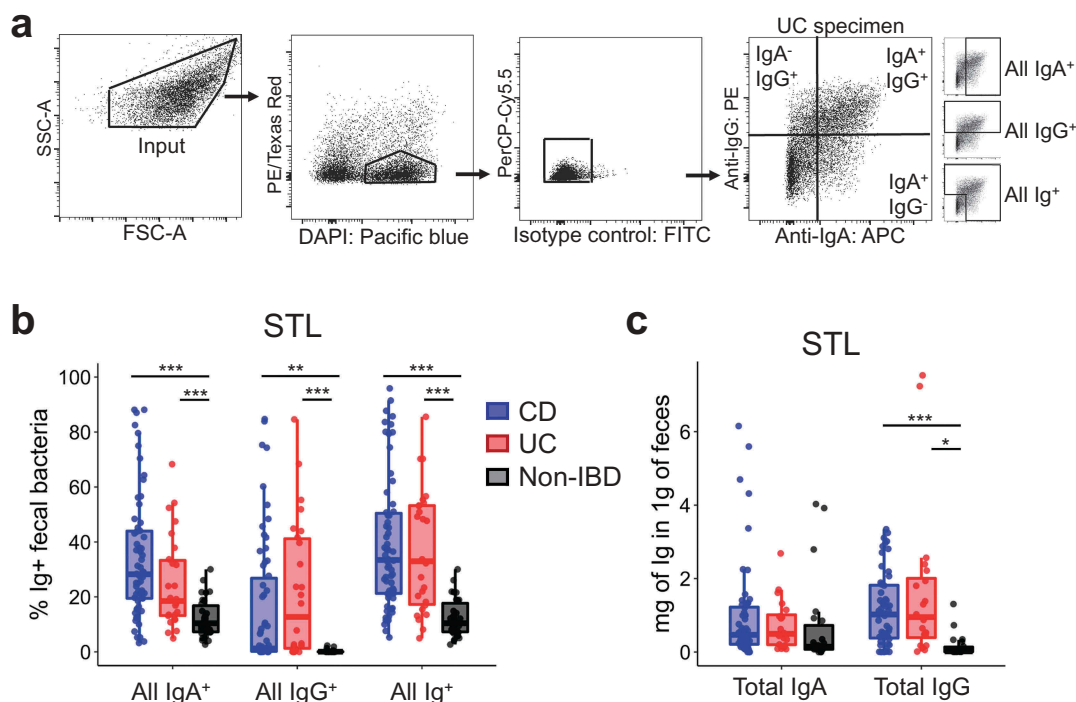


Figure 1. Increased Ig-bound fecal bacteria in IBD patients.

(A) Schematic of flow-cytometric bacterial sorting with representative UC specimen. All events that fall within the FSC and SSC gate were sorted as "Input." Bacterial events were gated based on DAPI staining and lack of auto-fluorescence (PerCP-Cy5.5 and PE/Texas Red) and isotype-control antibody staining. Depending on the presence of IgA⁺ or IgG⁺ events, two to four fractions were sorted from each sample: IgA⁻IgG⁻, IgA⁺IgG⁻, IgA⁻IgG⁺ and IgA⁺IgG⁺. (B) IBD patients show an increased percentage of IgA- and IgG-bound fecal bacteria. Data shown are the percentage of Ig-bound fecal bacteria in CD, UC, and Non-IBD fecal specimens from STL (62, 26, and 30, respectively). Each dot represents data from a single specimen, and boxes indicate the first and third quartiles (25th to 75th percentiles), and the whiskers extend from the box hinge to the largest or smallest value no further than 1.5*inter-quartile range (IQR). All IgA⁺ refers to IgA⁺IgG⁻ plus IgA⁺IgG⁺; All IgG⁺ refers to IgA⁻IgG⁺ plus IgA⁺IgG⁺; All Ig⁺ equals the sum of IgA⁻IgG⁻, IgA⁻IgG⁺ and IgA⁺IgG⁺. (C) Increased free fecal IgG, but not IgA, in IBD patients. Data shown are from STL cohort (52 CD, 20 UC, and 22 Non-IBD). *P*-values obtained from maximum likelihood two-tailed Student *t*-test of a linear mixed effect model with subjects as random effects. **p* < .05; ***p* < .005; ****p* < .0005, corrected for multiple testing with Benjamini-Hochberg.

Table 1. Subject demographics and treatment information.

Cohort	CD		UC		Non-IBD	
	STL	CBG	STL	CBG	STL	CBG
Number of patients	30	3	14	11	30	9
Number of specimens	62	8	26	26	30	11
% Male	33.3	33.3	57.1	63.6	33.3	55.5
Age	43.2 ± 14.8	46.0 ± 11.0	49.9 ± 15.5	38.5 ± 14.2	50 ± 15.5	44.9 ± 11.6
BMI (n)	25.7 ± 5.5 (28)	NA	29.2 ± 7.4 ^{##} (14)	NA	23.6 ± 5.2 (29)	NA
Race		100		100		100
% Caucasian	93.3 **		92.9		66.7	
% African American	6.7		7.2		20.0	
% Other	0.0 **		0.0		13.3	
Longitudinal (n)	20	1	6	6	0	1
Treatment [#]						
% Steroids (oral/IV)	26.7	66.7	20	72.7	0	0
% Vedolizumab	43.3	0	16.7	0	0	0
% TNF α inhibitors	36.7	0	10	0	0	0
% 5-ASAs	30	0	43.3	90.9	0	0
% Purine analogs	43.3	33.3	16.7	36.4	0	0
% Other immunomod [^]	10	33.3	16.7	27.3	0	0
% Antibiotics	13.3	33.3	3.3	9.4	7.2	0

Date presented as \pm mean.

[#]Treatment information for the second sample collected for subjects with longitudinal sampling. **CD vs Non-IBD; ^{##}UC vs Non-IBD

patient cohort differences (Table 1). Nonetheless, the increase in Ig⁺ fecal bacteria in two different geographic locations implies that variables associated with diet and environment are not essential to observe increased percentages of intestinal bacteria bound to IgA and IgG in IBD patients.

One potential explanation for the increased percentage of IgA- or IgG-bound bacteria would be an overall increase of Ig in the feces. To assess this, we measured the concentration of free-fecal IgA/IgG by ELISA and observed that free-fecal IgG was significantly increased in UC and CD subjects (Figure 1C), suggesting that the entry of IgG into the intestinal lumen, perhaps via barrier breach, may be an important factor leading to IgG-bound bacteria. By contrast, free-fecal IgA was not significantly different (Figure 1C), consistent with a previous report¹⁰. Thus, while the increased percentage of IgG-bound bacteria in IBD could be explained by an overall increase in IgG in the feces, that was not the case for IgA-bound bacteria.

Percentage of iga⁺ and igg⁺ fecal bacteria correlate with CD activity

As the range of Ig-bound bacterial frequencies in IBD overlapped with that in Non-IBD subjects, we asked whether disease activity correlated with the Ig-bound bacterial percentage. We assessed CD and UC

separately as they elicit distinct histological pathology and affect different regions of the gut. For CD, we observed a significant association between the percentage of all IgA- and IgG-bound fecal bacteria and disease activity by three disease activity scores, CDAI, HBI, and SIBDQ (Figure 2A, S2). This analysis was performed only for STL subjects as the CBG CD cohort was small. Notably, there was no clear correlation between free fecal IgA or IgG and % of Ig bound bacteria (Figure 2B), implying that increased bacteria-specific Ig, rather than the amount of fecal antibody, lead to the increased percentage of Ig-bound bacteria with CD flare.

We also observed similar relationships between IgA- and IgG-bound bacteria with certain laboratory markers of inflammation (Fig. S2). Overall, IgG-bound bacteria as compared to IgA-bound bacteria displayed better correlations with both disease activity indices and laboratory markers. Consistent with this, patient self-reported flare tracked with IgG-, but not IgA-, bound bacteria (Figure 2C; STL), which may reflect the patient perception of mucosal injury and bleeding into the stool. Moreover, the percentage of IgG⁺ bacteria to predict dichotomous CD disease activity (active vs inactive defined by CDAI <150 & HBI \leq 4) exhibited the highest area under the curve (AUC), which is a measure of separability of the test (Figure 2D). Together, these data show that increased Ig-bound fecal bacterial frequencies correlate with

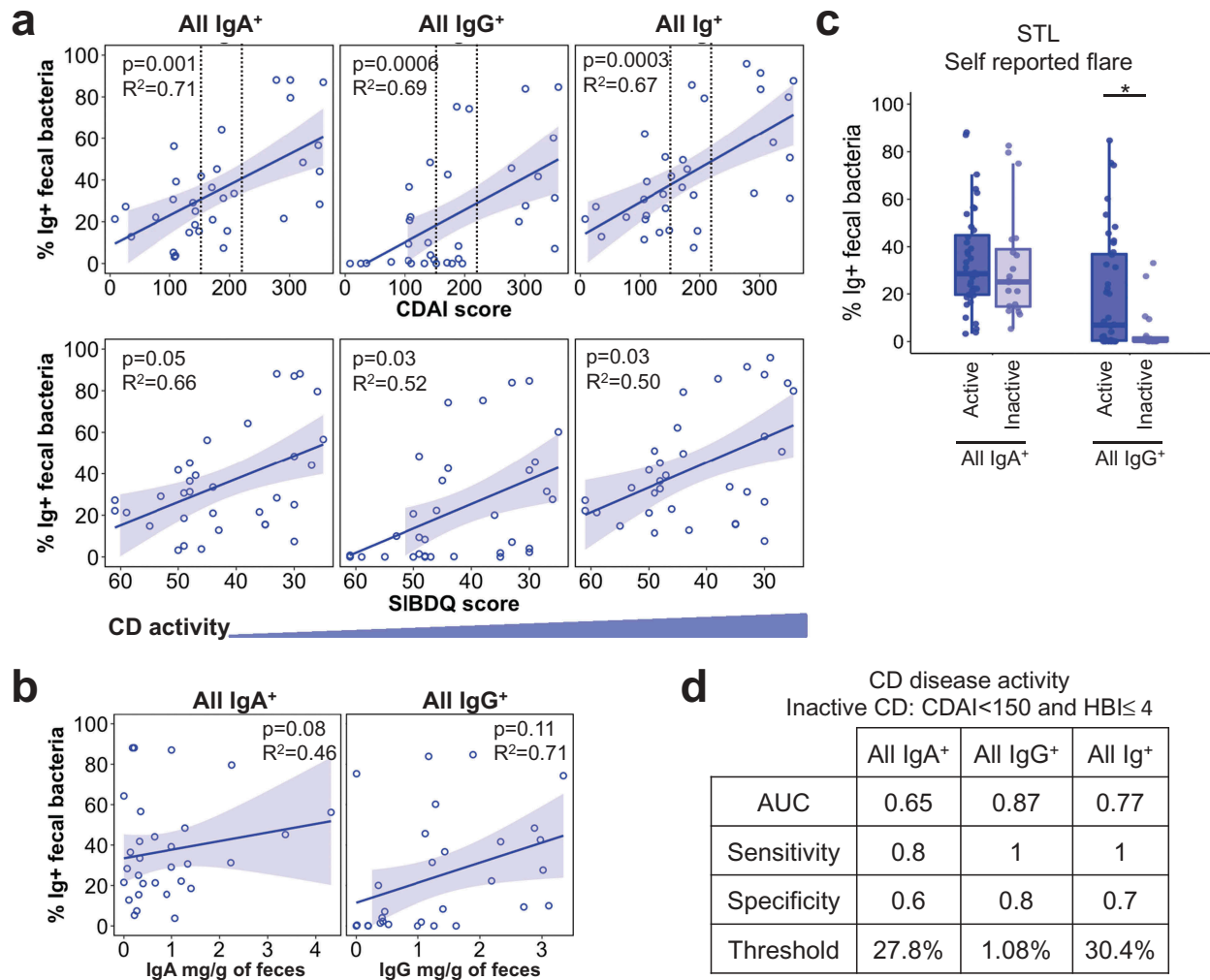


Figure 2. Percentage of IgA⁺ and IgG⁺ fecal bacteria correlate with CD activity.

(A) Percentages of Ig⁺ fecal bacteria correlate with CD disease activity measures CDAI and SIBDQ. Activity scores were measured at the time of fecal specimen collection in the STL CD specimens (CDAI n = 33, SIBDQ n = 33). (B) Percentage of IgA- or IgG-bound bacteria may trend towards correlation with total free fecal IgA or IgG, respectively, in STL CD specimens (n = 30). (C) Patient's self-assessment of CD flare (yes/no) is associated with the presence of IgG-bound fecal bacteria. Data shown are from self-reported 40 active and 20 inactive CD specimens from STL. (D) Utility of Ig-bound bacterial percentage for to discriminate between active vs. inactive CD. AUC, and sensitivity and specificity, for specified thresholds to determine inactive CD based on CDAI < 150 and HBI ≤ 4 (otherwise considered active) are shown from the STL cohort. *P*-values obtained from maximum likelihood two-tailed Student *t*-test of a linear mixed effect model with subjects as random effects. **p* < .05; ***p* < .005; ****p* < .0005, corrected for multiple testing with Benjamini-Hochberg.

higher disease activity and suggest that flow cytometry of fecal bacterial may be a useful biomarker for assessing disease activity in previously diagnosed CD patients.

Only IgG⁺ fecal bacterial percentage correlates with UC activity

We then asked whether disease activity in UC also affected Ig-bound bacteria. Similar to CD, we observed a significant positive correlation of all IgG⁺ and Ig⁺ bacteria percentage with UC disease

activity in STL patients based on quantitative measures such as partial Mayo, SIBDQ, and Physician's Global Assessment (Figure 3A, S3A). Laboratory markers like ESR, CRP, and % of basophils displayed similar correlations (Fig. S3A). The association of IgG⁺ bacteria with active UC was also seen with physician's assessments in CBG (Fig. S3B-C). In contrast to CD, however, the percentage of all IgA-bound bacteria was not positively correlated with UC disease activity (Figure 3A, S3A).

Similar to CD, there was no relationship between IgA-bound percentage vs. free fecal IgA in UC (Fig.

3C, 2B). In contrast to CD, UC patients show a stronger positive correlation between IgG-bound bacteria and free fecal IgG (Fig. 2B, 3C). This suggests that the degree of IgG entry into the lumen, presumably via barrier breach, has a more direct impact on the percentage of IgG-bound bacteria in UC. As expected based on these findings, a performance test for UC disease activity based on partial Mayo cutoffs (STL) or physician's reporting (CBG), yielded poor predictive power of IgA⁺ bacteria in discerning between active vs. inactive UC (Figure 3D). By contrast, IgG⁺ bacteria showed diagnostic potential in

discriminating between active vs. inactive UC at a threshold of 1.3% of fecal bacteria bound to IgG (Figure 3D). Thus, this analysis shows a notable difference between the IBD subsets: CD activity is reflected in IgA and IgG binding to bacteria, whereas UC activity appears to be defined primarily by IgG.

The Ig-bound and unbound bacterial repertoire are different

Although this study was not powered with a goal to identify Ig-bound bacteria in IBD, we analyzed

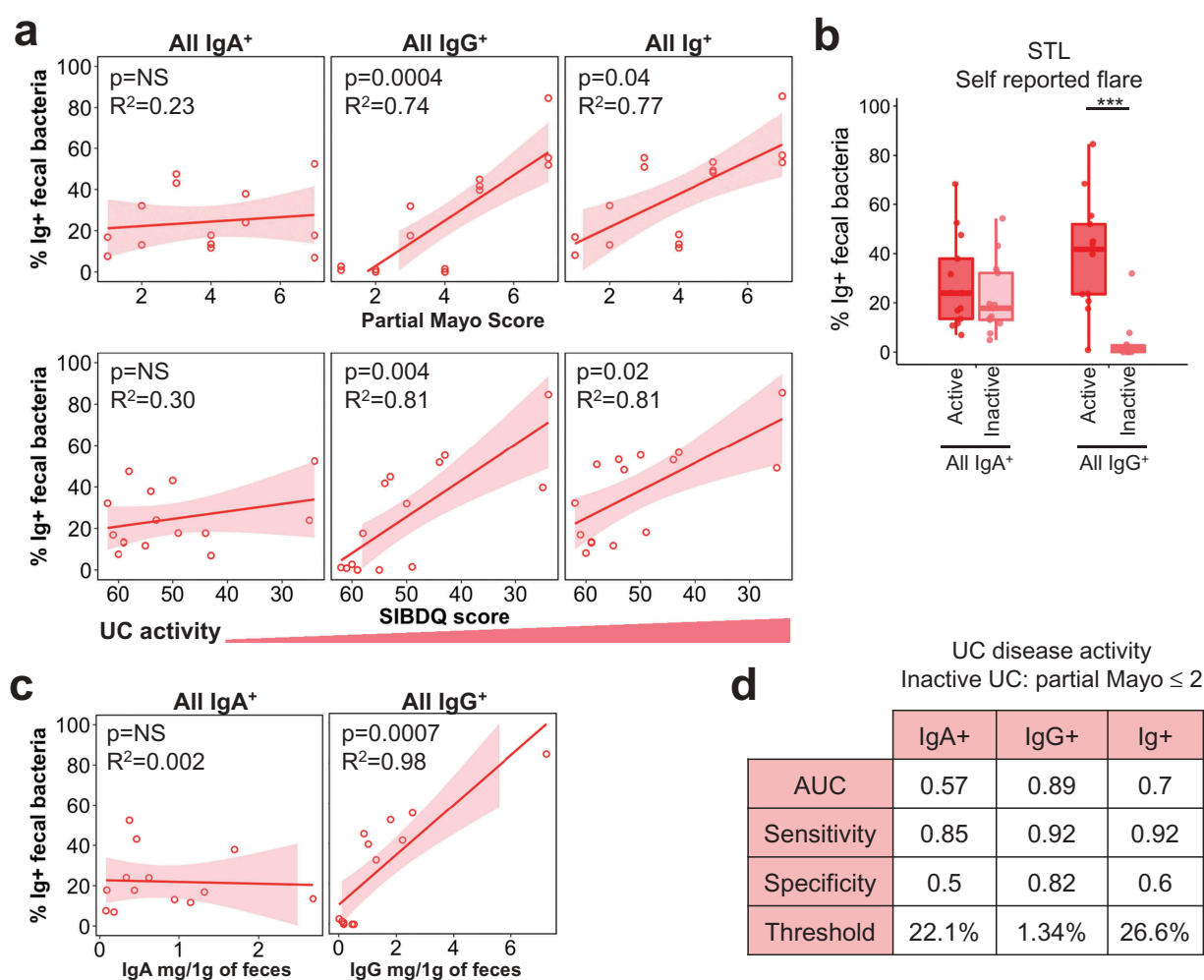


Figure 3. Only IgG⁺ fecal bacteria percentage correlates with UC activity.

(A) Percentage of IgG⁺ and Ig⁺, but not IgA⁺ fecal bacteria correlate with UC disease activity measures using partial Mayo score and SIBDQ. Activity scores measured at the time of fecal specimen collection in the STL CD specimens (partial Mayo $n = 15$, SIBDQ $n = 15$). (B) Patient's self-assessment of CD flare (yes/no) is associated with the presence of IgG-bound fecal bacteria. Data shown from 13 active and 13 inactive UC specimens from 14 patients in STL. (C) Percentage of IgG-bound bacteria correlates with total free fecal IgG whereas IgA-bound bacteria does not correlate with free fecal IgA in STL UC specimens ($n = 13$). (D) Utility of Ig-bound bacterial percentage for active vs. inactive UC. AUC, and sensitivity and specificity, for specified thresholds to determine UC disease inactivity based on partial Mayo ≤ 2 (otherwise considered active), are shown from the STL cohort. P -values obtained from maximum likelihood two-tailed Student t -test of a linear mixed effect model with subjects as random effects. $*p < .05$; $**p < .005$; $***p < .0005$, corrected for multiple testing with Benjamini-Hochberg.

the microbiota using 16S rDNA sequencing to determine whether we could detect changes in our limited sample set, which may be informative for future studies. First, we sequenced 16S rDNA from sorted events found in the forward and side scatter gates (FSC and SSC; **Figure 1A**) to assess microbial alpha and beta diversity. In both cohorts, UC or CD specimens displayed significantly lower alpha diversity using Chao1 (species richness) and Shannon (species richness and abundance) indices (Fig. S4A) in comparison with Non-IBD specimens, consistent with previous IBD studies using direct DNA extraction of fecal specimens.^{11–13} Beta diversity, or community differences, using the Unifrac metric was also different between the three disease groups by PCoA plots (Fig. S4B). Consistent with this, many OTUs displayed significant differences in overall percentage (Fig. S4C). Thus, 16S rDNA analysis of FACS-sorted bacteria from both cohorts showed characteristic features of microbial dysbiosis previously reported in IBD patients.

We next investigated if there were broad differences between the sorted Ig bound vs unbound bacterial populations. Notably, assessment of alpha diversity in STL patients using Renyi entropy, which includes Shannon diversity as order 1, did not reveal significant differences between the Ig⁺ and Ig⁻ fractions from a given disease group (**Figure 4A**). This suggested that there was no marked difference in species richness or evenness with Ig binding, which may be explained by the recent observation that many anti-bacterial IgA antibodies are polyreactive,¹⁴ potentially leading to considerable overlap in the Ig-bound and unbound microbiota. Although the Ig-bound and unbound fractions appeared to be of similar alpha-diversity, phylogenetic beta diversity at the operational taxonomic unit (OTU) level defined by >97% sequence identity, using the Unifrac metric between paired Ig-bound and unbound fractions from each specimen, was significantly different in each group (PERMANOVA $p < .001$ for CD, UC and Non-IBD). In addition, the Unifrac distance between Ig-bound and unbound bacteria for each sample was higher in CD vs Non-IBD and trended higher for UC vs Non-IBD patients (**Figure 4B**, $p < .01$, 0.08; respectively). For CD but not UC, the frequency of Ig-

bound bacteria positively correlated with the Unifrac distance between Ig-bound and unbound bacterial repertoires (**Figure 4C**), revealing another difference between these IBD subtypes. Thus, these data are consistent with previous studies in mice¹⁵ and humans,⁸ showing differences between the Ig-bound and unbound bacterial repertoire during homeostasis, which is increased during IBD consistent with heightened adaptive immune response against gut bacteria.

Strong correlation of IgA- and IgG-bound bacteria

To assess the enrichment of specific taxa in the IgA- or IgG-bound fraction, we calculated a log₂ ratio of the frequency of the taxa in the Ig-bound over unbound fraction, with positive values indicating a greater presence in the Ig-bound fraction, and a value of “0” indicating the equal presence in Ig-bound and unbound fractions. Log₂ ratios were arbitrarily capped at ~5.6 (49 fold change) to limit the effects of small or zero values in the numerator or denominator. We first assessed whether there is a relationship between the overall percentage for a given bacterial OTU in the sort input fraction and its enrichment in the Ig-bound fraction. Linear regression analysis between overall OTU percentage and all Ig⁺-enrichment for each disease subgroup revealed no consistent relationship with only a few OTUs with significant negative or positive slopes (Fig. S5A). Thus, Ig-enrichment is not, as a general rule, related to the relative frequency of the OTU.

Next, we wondered if IgA and IgG displayed preferential binding to OTUs since we observed differences in the frequencies of Ig-bound subsets by FACS in IBD subjects (**Figure 1B**, S1B). We did find OTUs that showed preferential binding by IgA or IgG in CD and UC (Fig. S5B). For example, *Akkermansia* and *E. coli* tend to be enriched in the IgA⁺ fractions (IgG⁺ or ⁻), and *Streptococcus* species were enriched primarily in the IgG⁺ fractions (IgA⁺ or ⁻). These findings suggest that the bacteria are targeted by both IgA and IgG but that one of the Ig classes may be limiting. There were also two OTUs in CD and two others in UC that were preferentially bound to the IgA⁻IgG⁺ fraction, suggesting that they triggered primarily an IgG

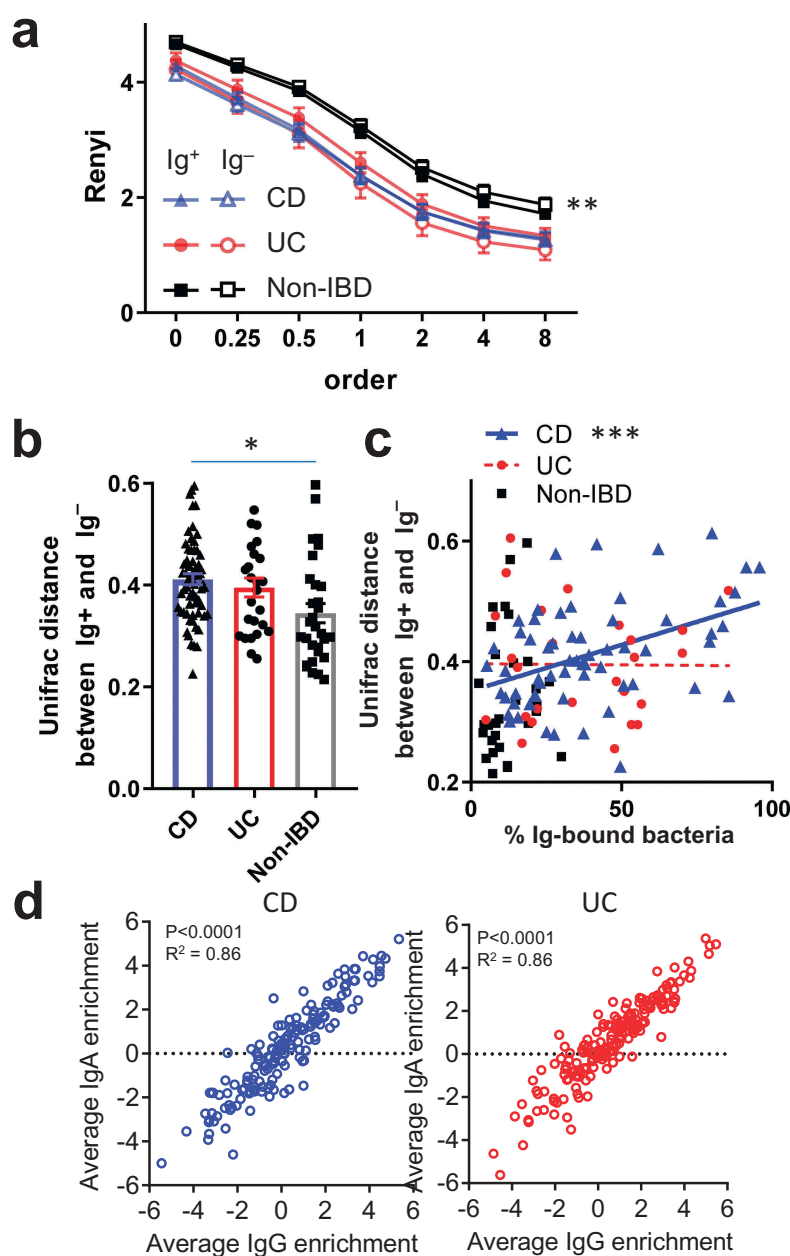


Figure 4. Characteristics of IgA and IgG-bound commensal bacteria.

(A) Similar alpha diversity of Ig⁻ and Ig⁺ sorted subsets of STL fecal specimens assessed using Renyi entropy, which assesses species richness (y-axis values) and evenness (slope). Non-IBD patients showed increased diversity (all $p_{adj} < 0.01$ except UC Ig⁺ vs Non-IBD Ig⁻ (0.06)). (B) Increased difference between Ig⁺ and Ig⁻ bacterial repertoires in IBD as assessed by Unifrac distance ($p_{adj} = 0.001$, 0.04 for CD and UC vs Non-IBD, respectively). (C) Unifrac distance between Ig⁺ and Ig⁻ bacterial repertoires correlate with Ig-bound bacterial frequencies in CD, but not UC by linear regression. (D) Most OTUs demonstrated a similar average IgA and IgG enrichment in CD and UC specimens. Pearson's correlation was calculated between the average IgA and IgG enrichment for a given OTU across all specimens in a disease group. Each dot represents a unique OTU.

response. While the differential Ig-binding of these OTUs corroborates the notion that CD and UC can elicit distinct mucosal immune responses to specific taxa, Pearson's correlations between IgA and IgG enrichment indices in CD or UC subjects

revealed that most OTUs displayed a similar enrichment index (Figure 4D). For this reason, we focused on the Ig⁺ fraction (IgA⁺ and/or IgG⁺) for the remainder of our analysis, as our data suggest that in general, IgG, if present, tends

to bind similar bacteria as IgA in both UC and CD.

Streptococcus spp. are Ig+ enriched in IBD compared with non-IBD patients

As the determination of OTUs that are preferentially Ig-bound is not well established in the literature, we utilized several methods (Figure 5A). One approach has been to identify the set of OTUs that are differentially bound to Ig (Ig enriched OTUs).⁷ We obtained the set of OTUs that were differentially found between the Ig-bound and unbound fractions in at least one patient group using lefSE,¹⁶ as well as DESeq2 to analyze count data between Ig+ and Ig⁻ fractions using the negative binomial model.^{17,18} (Figure 5B, S5C; S5D for STL+CBG data). A number of OTUs were identified as significant (or important) in only one statistical approach (Fig. S5C). For example, DESeq2 only identified one OTU (*Escherichia/Shigella coli*) as statistically significant in UC (STL) patients, whereas six OTUs were Ig-enriched using lefSE. As the statistical models differed, we utilized the intersection between these sets of OTUs to improve the selectivity of this Ig enriched OTU set (Figure 5B). We first noticed that there were five OTUs that were identified as Ig enriched in both IBD and non-IBD patients (bottom OTUs on Figure 5B; and Figure 5C), consistent with previous reports.^{7,8,19} These OTUs were not statistically Ig enriched in all patient groups by both lefSE and DESeq2, in part due to different sample sizes. Nonetheless, these data suggest that OTUs in the *Clostridium*, *Ruminococcus*, *Lachnospiraceae*, and *Escherichia* genera trigger an Ig response in both healthy individuals and IBD patients (Figure 5C).

We then asked if we could detect taxa that are uniquely Ig-bound in CD or UC as compared to healthy individuals. Seven OTUs were identified by DESeq2 or lefSE as Ig enriched in IBD but not Non-IBD patients (Figure 5B, D). Two of these OTUs (*Lactobacillus* and *Unc Peptostreptococcus*) appeared to be primarily found in CD patients and infrequently in UC and Non-IBD patients, suggesting that these bacteria expand with dysbiosis in CD and are

subsequently targeted by the immune system. We observed another group of bacteria which were common to both IBD and Non-IBD patients but were Ig enriched primarily in IBD (Figure 5D). These included *Actinomyces*, *Gemella*, two *Streptococcus*, and an *Unc Peptostreptococcus* OTUs that were enriched in IBD vs Non-IBD STL patients (Figure 5D; S5D for STL+CBG). Unexpectedly, these OTUs all encompass taxa that can be found in the oral pharynx.^{20,21}

The algorithm used above to select OTUs was UPARSE, which clusters related sequences and improves statistical power. However, closely related but non-identical 16S sequences may differ for Ig-bound and unbound bacteria and be clustered into one OTU. We, therefore, used dada2²² to identify amplicon sequence variants without clustering. This increased the number of OTUs which would result in loss of power with *fdr* correction. For example, the number of *Streptococcus* OTUs increased from 3 to 10 using dada2. Of the three dada2 OTUs found in >20% of patients (Fig. S5E), *S. salivarius* was still differentially Ig-bound in IBD patients. However, *S. mitis* (s12635) showed decreased bias to be Ig-bound with IBD vs. Non-IBD, whereas a new OTU, *S. australis* showed a preference to being IBD bound. Thus, the dada2 analysis confirmed that specific *Streptococcus* OTUs are Ig-enriched in IBD.

An alternative approach to identifying a set of Ig enriched OTUs is to compare directly between groups using Ig enrichment data ($\log_2(\text{Ig}^+/\text{Ig}^-)$).⁷ Using mixed effects modeling, there were several OTUs with a Benjamini-Hochberg adjusted $p < .25$ that demonstrated higher Ig-targeting in IBD specimens when comparing CD vs. Non-IBD and UC vs. Non-IBD specimens (Fig. S6). *S. salivarius* and the *Unc Peptostreptococcus* OTU was Ig enriched in IBD vs Non-IBD, corroborating the results from lefSE/DESeq2 above (Figure 5B). *Streptococcus salivarius* was the only Ig-enriched OTU that was identified for both CD and UC (both cohorts). OTUs with the same genus, but not species designations have been previously identified as uniquely targeted by IgA in IBD patients.⁷ Notably, all OTUs except *Dialister invisus* in the STL UC cohort were either not different in overall percentage or in some cases more frequent in Non-IBD fecal specimens as compared to UC or CD (Fig.

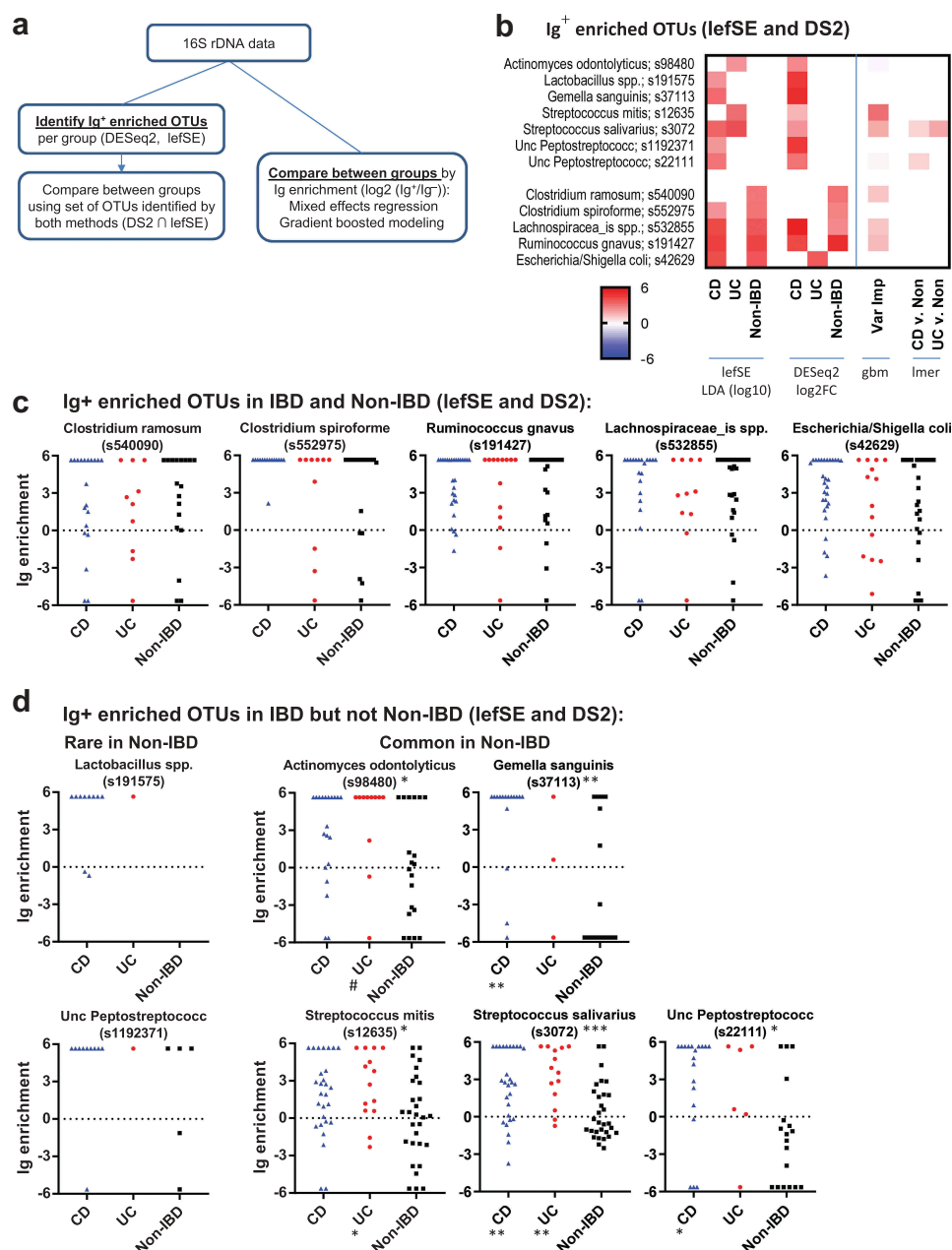


Figure 5. Taxa of Ig-enriched bacteria in IBD are often associated with oral pharynx.

(A) Schematic of methods used to identify Ig-enriched taxa in IBD. (B) Ig⁺ and Ig⁻ 16S rDNA data was analyzed for each patient group from STL. OTUs identified as Ig-enriched by both methods in at least one patient group is shown in the heat map. Data shown in the heatmap are LDA for lefSE, log₂ fold change for OTUs significant in DESeq2 (padj < 0.1), variable importance for gbm, and significance for lmer (* = 1, ** = 2). (C) OTUs identified by lefSE or DESeq2 as Ig-enriched in both IBD (CD or UC) and Non-IBD patients. Data shown are log ratios (Ig enrichment) of DESeq2 normalized counts. Each dot represents data from an individual patient. Kruskal–Wallis *p*-values between groups were not significant. (D) OTUs identified by lefSE or DESeq2 as Ig-enriched in IBD but not Non-IBD as per (C). Kruskal–Wallis *p*-values are shown # < 0.1, * < 0.05, ** < 0.005, *** < 0.0005. Symbols underneath the x-axis label indicate comparisons to Non-IBD.

S6), suggesting that increased percentage of an OTU due to dysbiosis during IBD does not typically explain increased Ig-targeting.

Finally, we tested the ability of the combined cohort Ig-enrichment data to predict IBD by performing

Gradient Boosted Modelling (GBM), a machine learning algorithm which may utilize interactions between OTUs to improve discrimination between CD, UC, and Non-IBD patients. The model was trained with 75% of the dataset and tested with the remaining 25%.

This revealed an average AUC of 81% in correctly identifying the right patient group (CD, UC, or Non-IBD) (Figure 6A). Assessment of the variables important for classification revealed a number of OTUs that were previously identified to be Ig-enriched by *lefSE* or *DESeq2* (Figure 5B, S5C). Notably, two of these include the two *Streptococcus* and one *Peptostreptococcus* OTUs that are preferentially Ig-bound with IBD. Thus, *gbm* analysis supports the notion that IBD alters the bacterial repertoire that is Ig-bound and implicates a greater potential role for *Streptococcus* spp. in the immune response during IBD than previously reported.

Ig-enrichment of specific OTUs correlates with frequency of ig-bound bacteria

To determine whether OTUs were preferentially Ig-enriched during active CD or UC, we performed correlations with various disease activity scores obtained for a subset of specimens in the STL cohort. Notably, the OTUs identified previously to be Ig-enriched in CD or UC often did not vary in Ig-binding with disease activity (Fig. S7A-B). Ig-binding to *S. salivarius* in UC, but not CD, may be associated with disease activity, but the number of UC patients with *S. salivarius* and a disease activity score was small. We did identify OTUs such as *Lactobacillus apodemi* that became more Ig-targeted with increased CD

disease activity (Fig. S7C). These OTUs were not originally detected in our CD vs Non-IBD analysis likely because of the range of Ig-enrichments in CD overlapped with the Non-IBD patients. Other OTUs were identified that became less Ig-bound with increasing disease activity (Fig. S7D). While we were able to identify some changes in Ig-binding with disease activity, the loss of power related to the number of patients with a quantified disease activity that also had a specific bacteria made this assessment difficult. We therefore asked whether Ig-binding of specific OTUs correlated with the log % of Ig+ bacteria as a surrogate for “immune activity in the gut.” Using data from all patients, three OTUs were significantly correlated (Figure 6B; *padj* <0.1), including the two *Streptococcus* OTUs discussed above, as well as *Lactobacillus apodemi* noted above (Fig. S7C). Thus, these data suggest that increases in immune activity in the gut based in Ig-bound bacteria do correlate with specific response to certain bacteria, particularly *Streptococcal* spp.

Discussion

In this study, we utilized bacterial FACS to study host:commensal interactions in IBD. We make the following observations: First, IBD patients have heightened adaptive immune responses against commensal bacteria as evidenced by increased

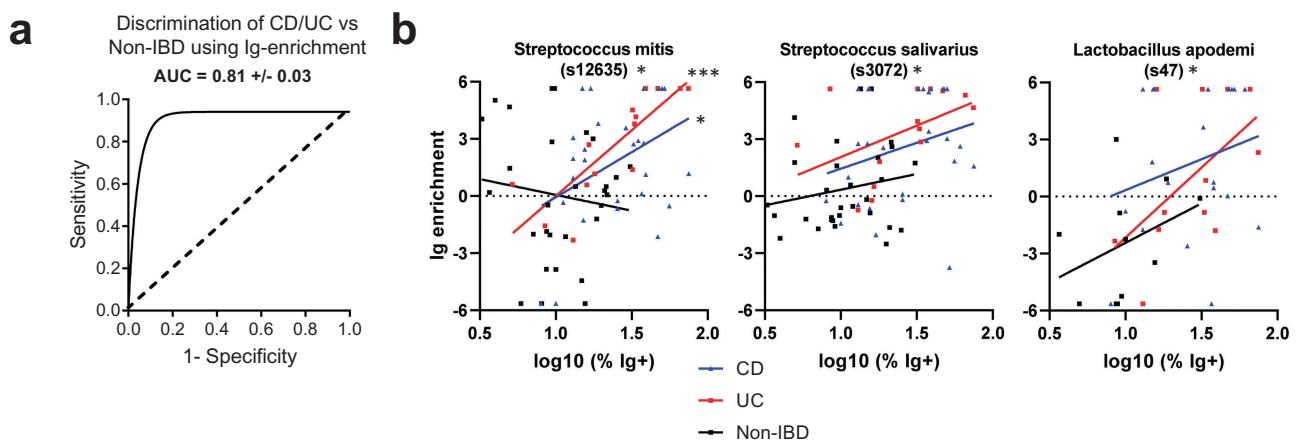


Figure 6. Bacterial OTUs enriched in IBD.

(A) ROC curve demonstrating the utility of Ig-enrichment data in differentiating between CD, UC and Non-IBD patients via gradient boosted modeling (GBM). Model was trained with 75% of Ig enrichment data for all CD, UC and Non-IBD fecal specimens and model accuracy was tested with the remaining 25% of data. Model accuracy and ROC curve generated over 10 iterations are displayed. (B) Significant correlation between Ig-bound bacterial frequency and two *Streptococcus* and one *Lactobacillus* OTUs based on linear regression of data from all patients. Lines represent linear regression of CD, UC, and Non-IBD patient groups. **p* < .05; ***p* < .005; ****p* < .0005, FDR corrected.

IgA- and IgG-bound bacterial frequencies compared with non-IBD individuals. Second, the percentage of fecal bacteria bound by Ig is directly correlated with IBD disease activity, although IgA-bound frequency correlates only with CD, but not UC, disease activity. Third, specific taxa such as *Streptococcus* show increased Ig-targeting in IBD compared with non-IBD patients and is correlated with the extent of the Ig response. Many of these findings were validated in two geographically separate cohorts. Thus, our findings support the hypothesis that mucosal immune tolerance to commensal bacteria is altered in IBD patients and suggest that flow cytometric analysis of fecal bacteria has potential utility for aiding the management of IBD.

Our data support the notion that adaptive immune responses against commensal bacteria are increased in IBD. Consistent with previous studies, we found that the percentage of IgA+ bacteria was increased in IBD patients.⁷ As this occurs without a corresponding increase in free fecal IgA, this implies that the fraction of bacteria-specific IgA increases in human IBD patients, a phenomenon that has previously been described in mice²³ In addition, we show that the IgG subset of immunoglobulin is also frequently bound to commensal bacteria in IBD patients. Notably, there were distinct differences in IgA and IgG binding to bacteria. Unlike IgA, IgG-bound bacteria are not found in healthy subjects. Moreover, the increased percentage of IgG+ bacteria is correlated with increased free fecal IgG in the intestinal lumen, whereby mucosal barrier breach might be an important mechanism leading to IgG, but not IgA-bound, bacteria. Thus, these data suggest that enhanced adaptive immune responses to commensal bacteria by both IgA and IgG are important characteristics of IBD.

Interestingly, the subtypes of IBD exhibit different patterns of Ig-bound bacteria. CD patients show increases in both IgA- and IgG-bound commensal bacteria with more severe disease, whereas UC patients show only IgG-bound bacteria. In addition, CD and UC show different patterns of IgA and IgG binding by flow cytometry, with UC often more dominated by IgG+IgA- tagged bacteria. We speculate that differences in the anatomic sites of gut inflammation and

histopathology between CD and UC account for the changes in the Ig-binding data. While future studies are required to understand the mechanistic basis for this observation, these data demonstrate that IBD subsets have distinct patterns of IgA and IgG responses to commensal bacteria.

These data also suggest that Ig-bound bacteria may be useful biomarkers for disease activity. Because of the differences in Ig-binding between UC and CD, the Ig-bound bacteria will likely need to be used in an IBD subset specific context. For example, the percentage of IgA-bound bacteria may be useful for monitoring CD as it strongly correlates with multiple clinical indexes of disease activity, whereas high IgA-bound bacterial frequencies in UC may indicate the disease state, but not activity. Notably, the presence of IgG-bound bacteria is strongly associated with patient perception of disease flare, particularly in UC, and may be useful in patients that under-report disease activity. Although future studies are required to correlate Ig-bound bacteria with histology on biopsy, the gold-standard assessment, these data suggest that Ig-bound fecal bacteria detectable by flow cytometry has the potential to be clinically useful as a surrogate biomarker for IBD disease activity.

In addition to the patterns of Ig-binding to bacteria, we analyzed the bacterial taxa bound to IgA or IgG during IBD. Although our sample size was in the range of the previous studies,⁷ we were not powered to robustly identify all the bacterial taxa that are Ig-bound in IBD as many taxa are not found in all patients. The previous study identified dozens of OTUs specific to IBD, but this may be without *fd*r correction. Techniques such as analysis of higher taxonomic levels commonly used in microbiome studies to decrease the effects of *fd*r correction were not particularly useful as Ig-binding could differ at the OTU levels. Despite these limitations, our data unexpectedly suggest that taxa often found also in the oral pharynx such as *Gemella*, *Peptostreptococcus*, and *Streptococcus*, are clearly targets of the immune response during IBD. While *Streptococcus* was identified as preferentially IgA-bound previously,⁷ our cohorts appear to contain *Streptococcus* in a much higher fraction of patients, being found in over 80% of patients. Contrary to other

studies,²⁴ we did not see a marked increase in *Streptococcus* frequency with IBD. Our data, therefore, suggest that the increase in Ig binding to *Streptococcus* is not related to the appearance of the bacteria with dysbiosis, but likely reflects the induction of an antigen-specific response during IBD. Notably, *Streptococcus* spp was predominant in the inflamed mucosa of CD patients in one study,²⁵ and was often found in intra-abdominal abscesses in perforating Crohn's disease²⁶ In addition to gastrointestinal sites of immune encounter, oral pharyngeal dysbiosis of *Streptococcus* and *Peptostreptococcus*²⁰ may also trigger an immune response. A prominent role for oral pharyngeal bacterial is notable in the context of a recent report suggesting that oral *Klebsiella* may be involved in IBD²⁷ Although we did not observe Ig-bound *Klebsiella* in our study, these data together suggest that immune interactions with oral bacteria may play an important role for IBD pathogenesis.

A pathogenic role for *Streptococcus*, however, has not been clearly defined. The Palm study included a *Streptococcus* species but was lost from the consortium in germ-free mice.⁷ In fact, other studies have suggested that *Streptococcus* can act as a probiotic that inhibit, rather than promote, colitis in murine models.²⁸ This observation may be consistent with the observation that *Streptococcus* does not readily elicit an Ig response in Non-IBD patients. However, the use of human isolates in mice^{29,30} as well as bacterial strain differences may account for these observations. Whole genomic sequencing of cultured isolates or metagenomic sequencing of Ig bound fractions may be useful to determine whether IBD patients harbor unique strains of *Streptococcus* that may facilitate immune recognition and disease pathogenesis. Future studies are therefore required to determine whether the immune response to *Streptococcus* or other bacteria also found in the oral pharynx simply represent a marker for the breakage of tolerance to gut commensals, vs an etiopathogenic role for these species in IBD.

Materials and methods

Cohort descriptions

Written informed consent was obtained from all study participants prior to inclusion in the study. Both

clinical groups collected detailed health information on all recruited subjects. On a subset of STL subjects, disease activity indices (CDAI, SIBDQ, HBI for CD; partial Mayo score, physician's global assessment, SIBDQ for UC) and serological test results were collected within four days of the associated fecal specimen. Averages for disease activity scores and subtypes of CD and UC patients based on Montreal behavior and location are detailed in Table S1. CBG IBD stool samples were designated active or inactive based on the physician's assessment often corroborated by the evidence of raised inflammatory markers or endoscopic inflammation. Non-IBD samples were from unrelated donors, except for two CD and seven UC patients of the CBG cohort, in which samples from Non-IBD 1st degree relatives were collected within 5 days of the fecal sample collection from the IBD-household member. Detailed metadata for all specimens are detailed in Supplementary File 1.

Subjects with any other gastrointestinal diagnosis or an intestinal resection were excluded from the study. Non-IBD subjects were verified to have no gastrointestinal disease diagnoses through patient survey and medical records. There were statistically significant differences in BMI and race in the STL, but not CBG, subjects. The distribution of age and sex in both cohorts was not statistically different. Disease activity score and disease subtype information is summarized in Table S1. Longitudinal sampling was performed for several subjects (see Supplementary File 1 for characteristics and medications at the time of each fecal specimen). All specimens were analyzed with correction for random effects of each subject through mixed effects regression modeling.^{31,32}

Bacterial FACS

A ~20 mg chip of stool was obtained from a stool aliquot stored at -80°C. The sample was weighed and dissolved in PBS at 25mg/ml by vortexing and sonication. After centrifugation, the supernatant was frozen for total IgA or IgG ELISA measurements for some samples. Spun down fecal material was suspended in 5mM N-acetyl-cysteine to break disulfide bonds in mucus and release bacterial cells. After two washes, the material was filtered through a 70µm filter and suspended in 20% FBS as a blocking reagent for 20 min. Samples were stained in 100 µl of 1:500 DAPI, 1:100 goat IgG

FITC – Isotype control for anti-IgA and IgG antibodies (Abcam 37374), goat anti-human IgA Dylight 650 (Abcam 98556) and goat anti-human IgG PE (Abcam 98596) for 30 min on ice, washed, and filtered through a 35 μm filter. Samples were analyzed by flow cytometry (FACSARIA IIu; Fig. S1A). For each sample, 30,000 events in the input, and Ig-bound and unbound fractions were FACS purified and concentrated to 5,000 events/ μl of sheath fluid for 16S analysis. Sort-purity for Ig-tagged bacteria was generally between 65% and 70%. To decrease contamination, ethanol was run through the cytometer during fluidics shutdown, and autoclaved PBS was used as sheath fluid.

16S rDNA analysis

A PCR reaction was set up in triplicate using 2 μl of concentrated bacteria each to amplify bacterial V4 hypervariable region of 16S rDNA using barcoded primers described previously³³ Sheath fluid was also sorted and sequenced separately to identify contaminant OTUs. Pooled PCR products were sequenced using the Illumina MiSeq platform (2 x 250-bp paired-end reads). OTU picking was performed using UPARSE (usearch v9, radius = 3%)³⁴ based on a calculated OTU frequency that combines the sequencing data from the FACS-sorted subsets at by the proportions indicated by flow cytometry. Dada2 v1.8²² was used to identify amplicon sequence variants of *Streptococcus* as noted in the text. OTU taxonomy was based on species designations with >97% confidence using Seqmatch, or from the Ribosomal Database Project (RDP v2.6)³⁵ classifier using default settings. The input sort fraction was used to calculate overall OTU percentages in each specimen.

All metrics for alpha diversity based on sequences rarefied to 3000 reads were calculated in QIIME and tests for significance were performed via mixed effects testing using the R package, *lme4* (v1.1–14).³¹ Beta diversity was calculated through *phyloseq* (v1.19.1)¹⁸ in R after rarefaction and pruning to remove OTUs that were present in three or less fecal specimens. After calculation of an unweighted unifracs distance matrix, significance was calculated using the *adonis* call in the *vegan* R package (v2.4–4),³⁶

with pairing either by Ig-subset or by same patient, as indicated.

Analysis of Ig-enriched taxa

Differential Ig-enrichment of a particular taxa is calculated as Log_2 (Frequency of taxa in Ig⁺ fraction/Frequency of taxa in Ig⁻ fraction). Ig-enrichment indices were arbitrarily capped at 5.61 (= log_2 (ratio of 49)) for taxa that were only present in the Ig⁺ fraction and absent in the Ig⁻ fraction, and -5.61 for taxa that were only present in the Ig⁻ fraction. We did not use the alternative approach to add a small fraction to the numerator and denominator to address the issue of division by zero in the ratio calculation, as we wished to use “NA” to indicate taxa that were entirely absent in a fecal specimen. To account for sequencing errors and imperfect bacterial sorting purity, for OTUs with total frequency of less than 1/5000 counts, no Ig-enrichment index was calculated. When comparing CD and UC Ig-enrichment OTU data to non-IBD individuals, all OTUs that were present in less than four fecal specimens were discarded to find generalizable trends. This log_2 ratio was arbitrarily capped at ± 5.61 (49 fold change) to limit the impact of values with a small denominator. As the composition of fecal microbiota can markedly differ between individuals, Ig-enrichment was left as an “NA” (not available) value for taxa absent in specimen. We did not use a 0 or an imputed value to resolve “NA” values, as it could bias towards similarity/difference and have variable effects on the analysis depending on the number of subjects which do not have those OTUs. We, therefore, probed the 16S rDNA data using methods more accepting of absent values (“NA”s) such as mixed effects testing, correlational analysis, and gradient boosted modeling. Gradient boosted models for Ig-enrichment data were trained with 75% of the fecal specimens and tested against the remaining 25% of the fecal specimens for all Ig enrichment data filtered as above, using the R packages *gbm* (v2.1.3) and *caret* (v6.0–77).³⁷ ROC curves and all associated information including AUC, sensitivity and specificity were calculated using the R package, *pROC* (v 1.10.0).³⁸

For identification of Ig-enriched OTUs, DESeq2 (v1.20)¹⁷ was used to analyze Ig⁺ vs Ig⁻ count data from STL (1st sample per patient) for CD, UC, or Non-IBD using `sftype = "poscounts"` to estimate dispersions. `lfeSE`³⁹ was also used to identify OTUs important in distinguishing between Ig⁺ or Ig⁻ fractions.

Free fecal Ig ELISA

IgG and IgA ELISA of fecal supernatant were performed in 96 well plates using the following antibodies and concentrations. Coating antibody: 1 µg/ml goat anti-human kappa (Southern Biotech (SB) 2061-01) for both IgA and IgG. IgA standard: SB 0155K-01 and IgG standard: SB 0150-01. Secondary antibody: 2 µg/ml peroxidase-conjugated anti-human IgA (Jackson ImmunoResearch 109-035-011) or anti-human IgG (H + L) (Jackson ImmunoResearch 109-035-088). Fecal Ig ELISAs were performed only on STL samples as CBG samples were available in limited quantities.

Statistics

Since we collected more than one stool sample from certain subjects, we performed a mixed effects regression analysis using `lme4` in R⁴⁰ and³¹ of the relationship between disease subgroup and various outcome measures (% of Ig⁺ bacteria, Ig enrichment, Chao1, beta diversity and most measures in the paper unless specified otherwise). As random effects, we had intercepts for subjects and as fixed effects, we had disease subgroups (UC vs. CD vs. Non-IBD, etc). *P*-values were obtained by maximum likelihood t-tests of the full model with the effect in question based on Satterthwaite's approximations. In situations where a mixed-effects model could not be generated, a generalized linear model (GLM), Mann-Whitney U or Kruskal Wallis testing was performed in R. FDR corrections using Benjamini-Hochberg procedure for all data were performed using the base stats package in R. Plots were generated using R package, `ggplot2` (v2.2.1)⁴¹ and Prism version 7.00 or 8.00 for Windows (GraphPad Software, La Jolla, California USA).

Patient Anonymity and Informed Consent

Stool specimens were collected with informed consent under institutional review board compliance at Washington University in St. Louis, St. Louis, USA and Addenbrooke's Hospital, University of Cambridge, UK.

Disclosure statement

No conflicts of interest to report.

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