Enteric bacteria are immune-reactive in patients with Crohn's disease with extraintestinal manifestations

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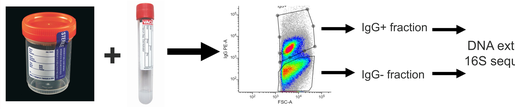
Background

- Enteric commensal bacteria contribute to local and systemic inflammatory disease; however, the interactions between commensals and host are complex, and the nature of those interactions is still not well explained
- Crohn's disease (CD) is a type of inflammatory bowel disease characterized by transmural inflammation of the ileum and colon, and can result in extraintestinal manifestations (EIM) of disease, including both peripheral (CD-SpA) and axial spondyloarthritis (CD-AxSpA).
- Alterations in abundance of enteric species have been associated with development of CD and CD-SpA[1], and specific enteric bacterial contributors to CD pathogenesis have been identified by flow cytometric sorting and 16S sequencing of bacteria recognized by mucosal IgA[2] (IgA-seq)
- We expand this technique by incubating fecal samples with autologous sera, capturing bacterial species recognized by circulating IgG in a process called IgG-seq
- **We hypothesize that these systemically-recognized enteric bacteria are linked to development of CD-SpA and CD-AxSpA**

Methods

IgG-seq: IgG-seq was conducted on 40 CD, 43 CD-SpA, and 14 CD-AxSpA samples, as well as on samples from 9 healthy control individuals (HCs), as per Figure 1 and previous work in our lab[2]. Samples were drawn from the MrCH-ON[3] and SMART cohorts. Once 16S sequencing was completed, sequences were processed with QIIME2, subsequent analysis was completed in R. Part of this analysis included calculation of immunoglobulin coating index (ICI)[4] at the level of genus (equation 1). Additionally, a simple ration of relative abundance in positive fraction to negative fraction was calculated for use in generating PCoA plots.



Stool sample + autologous serum

Figure 1: IgG-seq experimental schematic
Fecal samples were incubated with or without autologous serum, stained, and sorted into IgG-positive or negative fractions

$$ICI = -\frac{\log(Ig+) - \log(Ig-)}{\log(Ig+) + \log(Ig-)}$$

Equation 1: Immunoglobulin coating index (ICI): The ICI calculated in this manner normalizes this value to between -1 (taxon found exclusively in Ig-negative fraction and +1 (taxon found exclusively in Ig+ fraction). It provides a relative measure of immunoglobulin targeting of commensals.

Serum cell-free DNA sequencing: cfDNA sequencing was conducted by Dr. Iwijn de Vlaminck and his lab as per recent publication[5]
Bacterial strains and growth conditions: R. gnavus strain CC55 was graciously lent by Dr. Gregg Silverman. *R. gnavus* strains MrCH0305 and MrCH2147 were isolated from stool samples from participants in the MrCH-ON study, and strain identity was confirmed by 16S Sanger sequencing. Bacterial strains were routinely grown in brain-heart infusion (BHI, Anaerobe Systems) media overnight.
Western blotting: Cell pellets were lysed using BugBuster (Sigma-Aldrich) with lysozyme and protease inhibitors per manufacturer protocol. Aliquots of the purified membrane-enriched fractions were treated with Proteinase K (Sigma-Aldrich) per manufacturer protocol. Proteinase K treated and untreated samples were separated by SDS-PAGE and transferred to PVDF. IgG from sera samples was isolated using the Melon Gel IgG Spin kit (Thermo Fisher) per manufacturer protocol, and was used as primary antibody to probe membranes. HRP-tagged anti-human IgG was used as secondary, membranes were developed with Clarity western reagent (Bio-Rad), and imaged using Bio-Rad GelDoc and ImageLab

Results

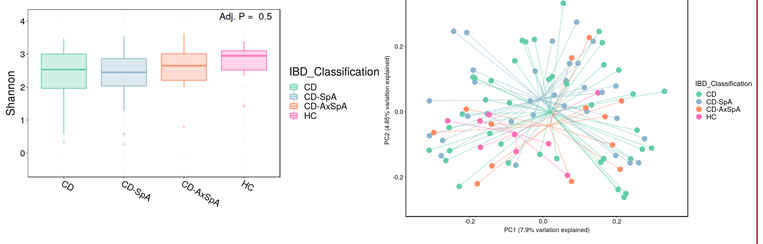


Figure 2: 16S sequencing of IgG+ fractions demonstrates no difference in alpha-diversity but significant difference in microbiome composition
Initial analysis of IgG+ fraction 16S sequences demonstrated no significant difference in alpha-diversity among HCs and CD with or without joint EIM. However, PCoA does demonstrate a significant difference in microbiome composition in these four groups (PERMANOVA P = 0.001). Of note, we observe a significant amount interpersonal variation in all groups.

Results

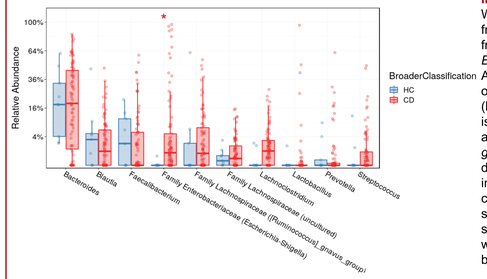


Figure 3: Relative abundance of genera vary in CD and HC samples
When comparing composition of the IgG-positive fraction in all CD patients to the IgG-positive fraction in HCs, a significant difference in *Escherichia-Shigella* abundance is noted. Additionally, a trend toward increased abundance of *Ruminococcus gnavus* group organisms (hereafter *R. gnavus*), as well as *Streptococcus*, is observed in CD as compared to HC. In additional analysis, *Escherichia-Shigella*, *R. gnavus*, and *Streptococcus* relative abundances demonstrated nominally significant increases in individuals with active SpA (BASDAI >4) as compared to those without active SpA. E. coli strains have been associated with CD(1,2), while streptococcal species have a known association with colorectal cancer. *R. gnavus* lipoglycan has been associated with lupus nephritis[6]

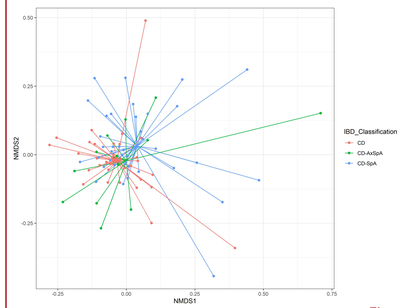


Figure 4: Significant difference in IgG ICI in CD with or without spondyloarthritis
IgG ICI was calculated at the genus level for all samples as noted in Equation 1, providing a measure of relative recognition of each genus. PCoA of IgG ICI demonstrates significant difference between CD, CD-SpA, and CD-AxSpA (PERMANOVA P = 0.012), thus indicating differences in recognition of enteric bacteria between these groups.

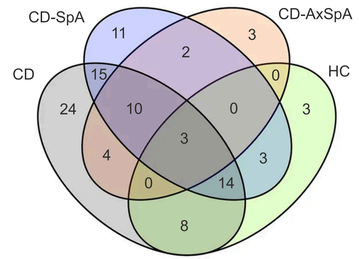
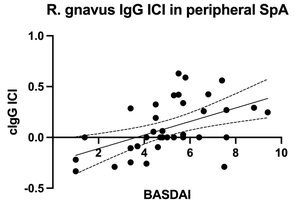
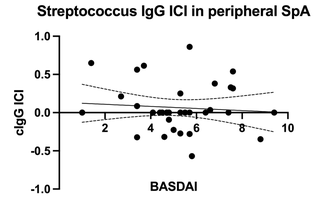


Figure 5: Highly recognized genera in HC and CD
In order to identify the genera that could account for the difference observed in composition, highly identified genera (those with ICI >0.2 in >1 subject in a specific group) in each subset were noted. The three genera highly recognized in all four groups were *Streptococcus, Pseudomonas*, and *Geobacillus* (which may represent contaminant[7]); those recognized only in CD-SpA and CD-AxSpA included *Blautia* and *Enterobacteriaceae*. Interestingly, *R. gnavus, Enterococcus*, and *Fusobacterium* were highly recognized in all CD groups, but not HCs.





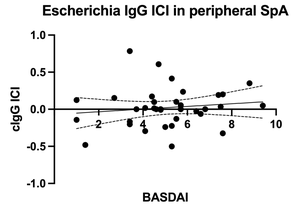


Figure 6: Correlation between BASDAI and IgG ICI in identified genera of interest.
R. gnavus IgG ICI demonstrates a positive correlation with BASDAI in patients with peripheral SpA (R^2 = 0.248, P = 0.002 for slope deviation from zero). While there is a mild positive association between *Escherichia* ICI and BASDAI, the slope of the correlation is not statistically significantly different from zero. Similarly, there is no significant association noted between *Streptococcus* ICI and BASDAI in patients with peripheral SpA.

Results

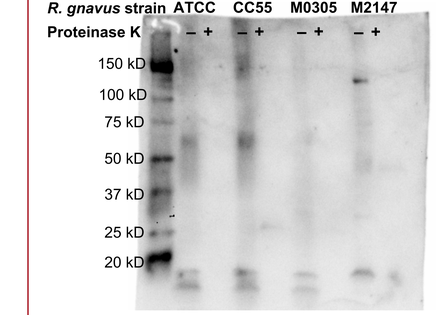


Figure 7: Western blotting of *R. gnavus* lysates shows recognition of a specific protein in a patient-derived strain
Recognition of specific *R. gnavus* antigens from different strains was tested using Western blotting, with IgG from patient sera used as primary antibody. The four strains are the ATCC *R. gnavus* reference strain; CC55, a strain from a healthy control patient, and M0305 and M2147, both isolated from patients with CD-AxSpA. This recognized 100-150 kD band is present only in an isolate from a patient with CD-AxSpA; it is not present in CC55 or in the ATCC reference strain. When lysates are digested with proteinase K, no band is observed. This specific band is recognized by multiple other CD and CD-SpA patients by Western blotting (representative image shown here).

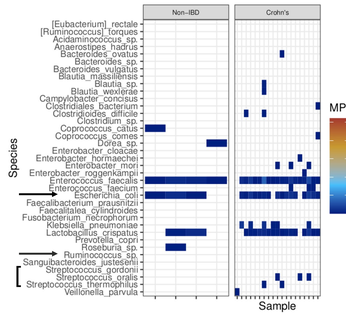


Figure 8: Cell-free DNA sequencing of CD and HC patients
cfDNA sequences found in serum demonstrate *E. coli* sequences in peripheral circulation in both HC and CD samples; however, no *Ruminococcus* sequences were detected in either group. Additionally, sequences from several *Streptococcus* species were noted in samples from CD patients, but not in samples from HCs. These results suggest that some genera of interest, such as *Escherichia* and *Streptococcus*, may translocate across the intestinal barrier, but *Ruminococcus* may not.

Conclusions and Future Directions

- Multiple enteric commensal genera are recognized by circulating IgG in CD, CD-SpA, and CD-AxSpA, and there is a significant difference in bacterial composition of the IgG-recognized fractions in these groups
- *R. gnavus* relative abundance and relative level of IgG recognition are positively correlated with severity of joint disease as measured by BASDAI
- *Streptococcus* and *Escherichia* cfDNA is found in circulation, but *Ruminococcus* sequences are not
- IgG from patient sera recognizes a specific *Ruminococcus* protein not present in all strains
- Further strains of *R. gnavus* from patients will be isolated, and whole-genome sequencing will be conducted on these strains
- Immunoprecipitation of *R. gnavus* lysates will aid in identification of the observed 100-150kD protein

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