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Infliximab Does Not Promote the Presence of Collagenolytic Bacteria in a Mouse Model of Colorectal Anastomosis

Sara Gaines¹ · Sanjiv Hyoju¹ · Ashley J. Williamson¹ · Jasper B. van Praagh^{1,2} · Olga Zaborina¹ · David T. Rubin¹ · John C. Alverdy¹ · Benjamin D. Shogan¹ · Neil Hyman¹

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Abstract

Background Previous work from our group has suggested a pivotal role for collagenolytic bacteria in the development of anastomotic complications. Tumor necrosis factor antagonists are a mainstay of treatment for patients with inflammatory bowel disease. The reported impact of these agents on key surgical outcomes such as anastomotic leak has been inconsistent. The objective of this study is to assess the impact of infliximab on the anastomotic microbiome in a mouse model of colon resection.

Design BALB/c mice underwent colon resection with primary anastomosis. Mice were randomly assigned to receive either an intraperitoneal dose of saline (control) or 10 mg/kg of infliximab for 8 weeks prior to surgery. On postoperative day 7, the animals were sacrificed. Anastomotic tissues were analyzed by histology with TUNNEL staining as a marker of epithelial apoptosis. In order to assess compositional and functional changes of the local microbiome, anastomotic tissues were further analyzed by 16S rRNA V4 region sequencing and for the presence of collagenolytic strains that may impair anastomotic healing. The main outcome measures were microbiome community structure and the presence of collagenolytic bacteria.

Results Infliximab-treated mice demonstrated an increase in epithelial apoptosis, consistent with the expected drug effect. Although infliximab modified the perianastomotic microbiome, no increase in the presence of collagenolytic bacteria was observed.

Conclusions Infliximab did not promote the emergence of collagenolytic bacteria or demonstrably impair anastomotic healing in a mouse model of colon resection and anastomosis.

Keywords Infliximab · Anastomotic leak · Microbiome

Introduction

Anti-tumor necrosis factor (anti-TNF) medications, such as infliximab (IFX), have become a mainstay of treatment for patients with IBD. In fact, the use of biologics in patients with Crohn's disease doubled from 2009 to 2015, from approximately 20 to 40%.¹ Biologic usage in a surgical cohort has been reported to be ~60%.² TNF inhibitors induce apoptosis of monocytes and T cells, thereby inhibiting cytokine release. The impact of this activity on wound healing and surgical complications has been an area of great concern and considerable controversy for many years.

Observational studies reporting the association of biologic medications with infectious postoperative complications such as anastomotic leak have generally been inconclusive. Numerous retrospective studies and several meta-analyses have been published with variable results, especially with respect to the impact on anastomotic complications. These studies are typically plagued by confounding selection factors, the timing of preoperative biologics, and uncertain/variable drug levels.³

Our laboratory has been interested in the role of bacterial collagenase production in the pathogenesis of anastomotic leak. Previous work from our laboratory has demonstrated that commensal strains of intestinal bacterial can be induced to produce high levels of collagenase during anastomotic surgery that can break down collagen 1 and 4 in an anastomosis and cause clinically significant anastomotic leaks.^{4,5} The aim of the present study was to determine the impact of IFX on the community structure and membership of the perianastomotic microbiome, and the presence of collagenase producing bacteria.

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Materials and Methods

Mouse Model of Colorectal Anastomosis

BALB/c male mice aged 8–10 weeks (Charles River) were used in all experiments. The animals were maintained in accordance with protocol IACUC 72491, with guidelines prepared by the University of Chicago Institutional Animal Care and Use Committee. Animals were allowed food and water ad libitum throughout all experiments. Five mice were randomized to treatment with 10 mg/kg of Infliximab (©Remicade, Janssen Biotech, Horsham, PA) in 3 cc normal saline through intraperitoneal injection using a 32G needle weekly for 8 weeks prior to surgery, and five control animals were administered 3 cc normal saline via intraperitoneal injection weekly for 8 weeks. All animals received the same perioperative care and intraoperative technique.

We used a previously validated mouse model of distal colon anastomosis.⁴ One hour prior to surgery, all animals received intramuscular cefoxitin (40 mg/kg Henry Schein, Mellville, NY, USA) and PO clindamycin (100 mg/kg Henry Schein, Mellville, NY, USA). All mice underwent general anesthesia with intraperitoneal ketamine (100 mg/kg Henry Schein, Mellville, NY, USA) and xylazine (10 mg/kg Henry Schein, Mellville, NY, USA). Under aseptic technique, all mice underwent laparotomy with transection of the colon at the peritoneal reflection. An anastomosis was created using 8-0 Prolene, and its integrity was tested by administering 100 μ L of normal saline via a 22-gauge blunt, olive tip needle enema. The abdomen was closed in two layers with 5-0 Vicryl followed by a 5-0 Nylon. Animals were resuscitated with 1 mL subcutaneous injection of 0.9% normal saline.

Postoperative analgesia consisted of subcutaneous injections of buprenorphine (0.05 mg/kg Henry Schein, Mellville, NY, USA) every 8–12 h for the first 48 h and a one-time subcutaneous dose of meloxicam (1 mg/kg Henry Schein, Mellville, NY, USA).⁵

Animal Tissue Analysis and Collection

Animals were sacrificed on postoperative day 7, and the peritoneal cavity was opened through the previous laparotomy scar. Anastomotic healing was graded using the previously described and validated anastomotic healing score (AHS): 0 = normal healing; 1 = loose adhesions; 2 = dense adhesions; 3 = abscess; 4 = gross leak with peritoneal contamination.⁴ The distal colon was resected proximally at the splenic flexure and distally at the anorectal junction in order to encompass the anastomosis for examination. The colon was opened longitudinally, and the luminal contents were removed. A portion of the distal colon was placed in 500 μ L of sterile normal saline for DNA isolation, and the remainder was placed in 10% glycerol for microbial culture analysis. In a subset of animals,

the distal colon and anastomosis were submitted for histological analysis (see below).

Histology and TUNNEL Staining

The distal colon including the anastomosis was submitted for histological analyses according to standard protocol (fixed in 10% formalin for 24 h followed by washing in 70% Ethanol). Embedded tissue was cut in 5- μ m slices and subjected to TUNNEL staining to detect apoptosis, consistent with the expected microscopic findings in IFX-treated animals.

Microbial Culture for Collagenase Activity

Homogenized colon tissue aliquots were thawed and serially diluted. Twenty microliters of the stock solution and each dilution was plated onto skim milk-containing Enterococcosel™ agar (15% skim milk, US Biological Life Sciences, Salem, MA, USA), and MacConkey (Difco™ MaConkey, BD, Sparks, MD, USA) (20% skim milk) was prepared as previously described.⁶ The CFU count was normalized to sample weight.

DNA Isolation

The collected mouse samples were homogenized with the FastPrep-24™ 5G Instrument (MP Biomedicals, Solon, OH, USA). Total DNA was extracted from 300 mL of homogenate with the FastDNA SPIN KIT (MP Biomedicals, Solon, OH, USA) according to manufacturer's protocol.

16S rRNA Gene Analysis

Total DNA isolate obtained from the mouse samples was used as template DNA. Each 25 μ L PCR reaction contained 12.5 μ L of AccuStart II PCR ToughMix (Quantabio, MA, USA), 8.5 μ L of PCR-grade water, 1 μ L forward primer, 1 μ L mPNA (50 μ M) (PNA BIO INC, CA, USA) to block host target mitochondrial contamination, 1 μ L Golay Barcode Tagged Reverse Primer (5 μ M), and 1 μ L of template DNA. The PCR conditions were 94 °C for 3 min followed by 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s with a final extension of 10 min at 72 °C. The resulting amplicons were subsequently cleaned, pooled, and quantified using the Quant-iT PicoGreen double-stranded DNA assay kit following EMP-benchmarked protocols (<http://www.earthmicrobiome.org/emp-standard-protocols/>). Pooled amplicons were then pair-end sequenced on Illumina MiSeq sequencing run at the Argonne National Laboratory, IL, USA.

Bacterial 16S V4 region (515F–806R primer pair) sequences were processed using Qiime2 (v2018.11).^{7–9} Default parameters were used for demultiplexing. Quality control was performed with the DADA2 function integrated

in Qiime2 to truncate forward and reverse reads, to denoise the data and for detection and removal of chimeras.¹⁰ The representative sequence variants of each sample were retained and assigned to bacterial taxa using a Naive Bayes classifier trained on the Greengenes 13_8 99% OTUs.¹¹

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software. Unpaired Student's *t* tests were used for comparisons between two means. Mann-Whitney nonparametric test was used when there was no assumption of normal distribution.

Results

Infliximab Induces Epithelial Apoptosis

Anastomotic healing appeared to be similar in both groups (Fig. 1a). Two out of four mice in each group had minor adhesions. One mouse in each group died from anesthetic complications immediately post-operatively (Fig. 1b). One mouse in each group had dense adhesions surrounding the anastomosis. One mouse in each group developed an abscess (Fig. 1c) suggestive of a significant anastomotic complication. Using histological TUNNEL staining, the distal colon and anastomosis in animals treated with IFX showed more epithelial apoptosis compared with animals treated with normal saline alone (Fig. 1d).

Treatment with Infliximab Does Not Promote Perianastomotic Colonization with Collagenase Producing Organisms

There was no difference in colonization with gram-negative bacteria between animals treated with IFX and only one animal-harbored bacterium capable of collagenolytic activity

($p > 0.05$, Mann-Whitney test) (Fig. 2a). There were no enterococcal species capable of producing collagenase recovered from the anastomotic tissue from either treatment group ($p > 0.05$, Mann-Whitney test) (Fig. 2b).

Infliximab Alters the Anastomotic Microbiome with an Emergence of *Turicibacter*

Using 16S rRNA analysis, the compositional changes in the anastomotic microbiome revealed the appearance of *Turicibacter* at the genus level in two out of four animals treated with IFX. One animal harbored *Ruminococcus* which was not present in the control group. The control group demonstrated relative stability in proportions of bacteria except in one animal in which *Akkermansia* dominated (Fig. 3).

Discussion

Results of the present study indicate that although IFX alters the local microbiome after colon resection, it does not promote colonization with collagenolytic bacteria at a dosage commonly used in clinic practice. The colonic mucosa exhibited evidence of apoptosis on TUNNEL staining as compared with the saline-infused animals, suggesting adequate absorption and suggesting that the drug had the expected effect on the colonic epithelium.

We sought to assess the impact of IFX on the perianastomotic microbiome, given out previous work demonstrating a key and causative role for collagenolytic bacteria in the pathogenesis of anastomotic leak.⁵ We found no increase in the presence of collagenolytic bacteria in the IFX-treated group. However, IFX did alter the microbiome of the experimental group with a notable emergence of the genus *Turicibacter*. While the impact of the microbiome is largely unknown, it is notably depleted in animal models of inflammatory bowel disease.^{11,12} Further work will be needed to assess the role of this organism.

Fig. 1 **a** Upon inspection of the anastomosis at sacrifice, there was no difference in anastomotic healing scores between the control group and the animals treated with infliximab with a mean of 1.75 in each group ($n = 5/\text{group}$, $p > 0.05$, unpaired *t* test). **b** Representative image of a score of 1 on the anastomotic healing scale characterized by minor adhesions

adjacent to the anastomosis. **c** Representative image of a healing score of 3 with the image depicting abscess formation at the site of anastomosis with dilation of the proximal colon suggestive of a contained anastomotic leak. **d** TUNNEL staining (brown) demonstrated an increase in apoptosis in infliximab-treated mice

of complications. If biologics cause an increase in complications after surgery, one might expect that higher drug levels would be associated with more complications. This has not generally been the case, and many patients treated with biologics have no discernable drug in their system.³⁰ In addition, there does not appear to be any relationship between the timing of biologic administration and the complication rate.^{32,33} Again, one might predict that closer proximity to the time of surgery would increase the complication rate; again, this does not appear to be the case.

In addition, interpretation of the available data has been complicated by selection factors and confounding. Patients are not “randomly” assigned to receive a biologic, and patients who receive these agents may be sicker or have a longer delay to surgery, with relative depletion of their physiologic reserve. The lack of clarity on this issue has led to uncertainty in deciding the optimal management of biologics in patients who undergo surgery.

Practice patterns may have a significant impact on patient care with potential for unintended harm. It is often advised that patients wait for two drug half-lives to pass before undergoing surgery, in order to allow the medication to clear.^{34,35} Others have suggested a more liberal use of fecal diversion in patients with Crohn’s disease based on biologic exposure.³⁶ As such, studying the potential impact of biologics on anastomotic complications is a critical issue impacting the surgical management of patients with IBD.

Despite histologic evidence of the expected drug effect at the anastomosis, there was no grossly obvious impact on the anastomotic healing score at sacrifice. The anastomotic healing score has previously been validated and provides a reliable measure of anastomotic healing.⁴ Clearly, a much larger sample size would be required to meaningfully assess the impact of IFX on anastomotic healing in the murine model. Further, the applicability to humans, especially IBD patients who have so many longitudinal factors that may impact their microbiome, is uncertain. In future studies, it may be important to humanize the mouse gut with a microbiome more typical of that of an IBD patient.

Further limitations of our study include the use of a single dosage of IFX; it may be that different doses or intervals may impact anastomotic healing differently. We again acknowledge that a larger sample size could have modified the findings, especially regarding anastomotic healing and no conclusions on this clinically important issue may be appropriately drawn.

In summary, we found that IFX did not promote the emergence of collagenolytic bacteria in a murine model. There is a clear need for basic research to try and “unpack” the varied influences of biologics on the intestinal epithelium and the gut-associated microbiome, considering the critical implications the drugs may have for patients undergoing surgery for IBD.

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Compliance with Ethical Standards

Competing Interests Dr. David Rubin is a Consultant for Janssen, AbbVie, Merck, Pfizer, Takeda, Amgen, and Samsung Bioepis, and receives research support from Takeda. All the other authors declare that they have no conflict of interest.

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