Colonic Immunopathogenesis of *Clostridium difficile* Infections

Running Head: Gut Inflammation and *C. difficile*

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FOOTNOTES

CONFLICT OF INTEREST

The authors have no conflicts to report.

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There are major gaps in our understanding of the immunopathogenesis of *C. difficile* infections (CDI). In this study, 36 different biomarkers were examined in the stools of CDI and non-CDI patients using the Proteome Profiler Human Cytokine Array assay and quantitative ELISA. CDI diarrheal stools showed higher relative amounts of the following inflammatory markers than the CDI-negative diarrheal stools: C5a, CD40L, G-CSF, I-309, IL-13, IL-16, IL-27, MCP-1, TNF-α, and IL-8. IL-8 and IL-23 were present in a larger number of CDI diarrheal stools compared to the CDI-negative diarrheal stools. Th1 and Th2 cytokines were not significantly different between the CDI and non-CDI diarrheal stools. Lactoferrin and calprotectin concentrations were also higher in the CDI stools. Our results demonstrate that CDI elicits a pro-inflammatory host response and report for the first time, IL-23 as a major marker in CDI stools. IL-23 may explain the lack of robust immunological response exhibited by a proportion of CDI patients and may relate to recurrence; IL-23 levels induced during CDI in these patients may be inadequate to sustain the cellular immunity conferred by this cytokine in promoting the induction and proliferation of effector memory T cells.
INTRODUCTION

*Clostridium difficile* infection (CDI) is the leading cause of infectious antibiotic-associated diarrhea and a major problem for the elderly and immunocompromised (1). The incidence of CDI has more than doubled since 1996, making CDI the most common healthcare-associated bacterial infection in many hospitals in the United States (2). One of the hallmarks of CDI is inflammation of the colonic mucosa due to the actions of toxins A and B, which are released by this bacterium during infection (3). The toxins act on the actin filaments of colonic epithelial cells, which disrupts tight junctions, induces apoptosis, and causes infiltration and aggregation of neutrophils (4, 5).

Recruitment and aggregation of neutrophils is an early significant event in CDI, which in severe cases, can lead to pseudomembranous colitis (6, 7). Only 28-63% of stools from CDI patients show high neutrophil counts (8), in spite of the inflammatory nature of the infection. As a result, other leukocyte-derived products excreted in feces such as cytokines, lactoferrin, and calprotectin have emerged as candidate biomarkers of intestinal inflammation (9, 10). Fecal lactoferrin is elevated in highly inflammatory enteric diseases, but not in healthy controls (6). The increase in fecal lactoferrin during intestinal inflammation is proportional to translocation of neutrophils into the gastrointestinal tract (11). More than 60% of the total cytosolic protein content of neutrophils is calprotectin. This calcium and zinc-binding protein plays a regulatory role in inflammation with antimicrobial and antiproliferative properties (12). Fecal calprotectin levels correlate with fecal excretion of neutrophils (13). Fecal lactoferrin and calprotectin levels help predict increased translocation of granulocytes into the intestinal mucosa.
In this study, we sought to examine the immunopathogenesis of CDI by analyzing the major inflammatory markers present in the stools of CDI and non-CDI antibiotic-associated diarrheal patients and hospitalized controls without diarrhea. This was done with the goal to establish the expression pattern of these biomarkers during CDI to help provide insight into the immunologic pathogenesis of CDI.

MATERIALS AND METHODS

Patients Population. Stool samples were collected from patients enrolled for an ongoing study approved by the Institutional Review Board of The University of Texas Health Science Center at Houston, Texas (14). All of the participating patients or their legal guardians provided written informed consent upon admission to the hospital. The patients were enrolled at a 700-bed university hospital in the Texas Medical Center in Houston, Texas, whose physicians ordered a stool examination for CDI from December, 2010 to June, 2011.

Stool Samples. A total of 100 antibiotic-associated diarrheal stool samples from 100 different patients (50 CDI-positive, 50 CDI-negative) and 45 diarrheal stools from hospitalized control patients without diarrhea were analyzed. The stools were collected on the first day the patients reported to the hospital and stored at -80°C until tested. Stool samples were tested by the tissue-culture cytotoxicity assay and cultured on C. difficile-selective media (CCFA) and the CDPA plates (15), followed by PCR for the presence of the toxin genes (tcdA and/or tcdB). All of the stool samples classified as CDI-negative were further tested and confirmed to be negative for the C. difficile toxins.
using the Wampole C. difficile Tox A/B II Assay (Techlab, Blacksburg, VA). The CDI-positive and CDI-negative samples were matched by date of collection. The control stools were negative for C. difficile by the real-time BD GeneOhm Cdiff PCR assay for the tcdB gene, toxigenic culture, and ELISA for toxins A and B.

Cytokine Assays. A total of 50 CDI-positive and 50 CDI-negative diarrheal stools were initially evaluated for the presence and relative amounts of 36 different inflammatory markers using the Proteome Profiler Human Cytokine Array Panel A Kit (R&D Systems, Minneapolis, MN). The Proteome Profiler Human Cytokine Array assay was performed based on the instructions provided by the manufacturer. Stools from the hospitalized controls without diarrhea were not evaluated by this initial assay, but were included retrospectively in the quantitative ELISA (described below) for comparison. Briefly, 300 mg of each stool sample was thoroughly suspended in 1.5 ml of Array Buffer 5, incubated at room temperature for 15 min, and centrifuged for 5 min at 5,000 x g. The supernatant (1 ml each) was added to a cocktail of biotinylated antibodies and incubated at room temperature for 1 hr. The sample-antibody mixture was subsequently incubated at 4°C for 19 hrs with a membrane embedded with antibodies specific to each of the 36 different inflammatory markers analyzed. Following three washes, 3 ml of a 1:1000 dilution of streptavidin-HRP was added to each membrane and incubated at room temperature for 35 min. For detection, the membranes were probed with the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and exposed to an X-ray film. The exposed film was processed using SRX-101A Medical Film Processor (Konica Minolta). The pixel densities of each blot (band) representing the
amount of each inflammatory marker present were determined using ImageJ software (National Institutes of Health, Bethesda, MD).

All the images from the 100 arrays were normalized by subtracting the background and inverted to eliminate the background differences. To measure the pixel density, a fixed size rectangular box was generated around each dot blot/band and the pixel density was measured. The same sized rectangular box was used for all the bands in all the 100 arrays performed. For analysis, the pixel densities of the negative control on each array were subtracted from the pixel densities obtained from each band on the array. The data was further converted and normalized into fold change in expression by dividing the pixel densities of each of band by the average pixel densities of the Streptavidin-HRP Reference Spots located at the three corners of each array.

Quantitative ELISA Assays. The fecal concentrations of IL-8, IL-23, TNF-α, IFN-γ, and IL-13 were determined by quantitative ELISA (R&D Systems, Minneapolis, MN), using the instructions provided by the manufacturer. The relative amounts of these cytokines were determined by the initial Proteome Profiler Human Cytokine Array assay to be significantly different between the CDI-positive and CDI-negative diarrheal stools. The concentrations of these cytokines were determined from 50 CDI-positive, 50 CDI-negative, and diarrheal stools from the 45 hospitalized controls without diarrhea.

Lactoferrin and Calprotectin Assay. The concentrations of lactoferrin and calprotectin present in 50 CDI-positive, 50 CDI-negative, and 45 diarrheal stools from hospitalized controls without diarrhea were determined. Fecal lactoferrin was measured using the
IBD-SCAN (Techlab, Blacksburg, VA) with 50 mg of each stool. Fecal calprotectin was
determined using the HK325 Human Calprotectin ELISA Kit (Hycult Biotech, Plymouth
Meeting, PA). Briefly, 100 mg of each stool was extracted in 5 ml extraction buffer and
the supernatant was incubated in 96-well microtiter plates coated with human
calprotectin-specific antibody for 1 hr at 25°C. A biotinylated tracer antibody was added
to detect the calprotectin that bound to the antibodies coated on the plate surface. Then,
streptavidin peroxidase conjugate was added. Following incubation, the unbound
conjugate was washed and tetramethylbenzidine was used as the detection substrate

Statistical Analysis

The data were statistically analyzed by Mann-Whitney two-tailed non-parametric test of
significance and One-Way ANOVA using GraphPad Prism version 5.02 for Windows
(GraphPad Software, San Diego, CA).

RESULTS

A total of 50 stools each from CDI-positive and CDI-negative antibiotic-associated
diarrheal patients and 45 stools from hospitalized controls without diarrhea were
collected and examined. The average ages among the study groups were 58, 56, and
63 years for CDI-positive, CDI-negative, and control patients without diarrhea,
respectively. The majority of the patients enrolled were from three major ethnicities:
White/Caucasians, Black/African-Americans, and Hispanics/Latinos (Table 1). No other
major co-morbidities were noted among the study population.
One of the clinical hallmarks of *C. difficile* infection (CDI) is colonic inflammation, which is mediated generally by cytokines and other pro-inflammatory proteins such as the toxins A and B. To assess the essential inflammatory biomarkers that are elicited during CDI, the fecal amounts of cytokines, chemokines, and other acute proteins were examined from stools collected from diarrhea and non-diarrhea patients. The CDI-positive and CDI-negative diarrheal stools were initially tested for the presence and relative amounts of key inflammatory biomarkers using the Proteome Profiler Human Cytokine Array Panel A Kit (R&D Systems, Minneapolis, MN). This assay simultaneously detects 36 different inflammatory biomarkers present in a stool sample. A representative image showing the bands (dot blots) indicative of the inflammatory biomarkers detected in the samples is shown in Figure 1. The intensities of the bands differed within and between the different stools evaluated, suggesting different amounts of the biomarkers present in the stools. Some of the 36 inflammatory markers tested were not detected, but the total number of different inflammatory biomarkers detected was also different between the groups of stools examined.

The intensities of the dot blots were empirically determined and expressed as pixel densities using the ImageJ software (National Institutes of Health, Bethesda, MD). Following normalization, the relative band intensities representing the relative amounts of all the inflammatory markers detected in the CDI-positive and CDI-negative diarrheal stools are shown in Figure 2. The CDI-positive stools exhibited significantly (P<0.05) higher relative amounts of the following biomarkers compared to the CDI-negative
diarrheal stools: C5a, CD40L, G-CSF, I-309, IL-13, IL-16, IL-27, MCP-1, TNF-α, and IL-8. On the other hand, the relative amount of IL-23 was significantly higher (P< 0.05) in the CDI-negative stools than that of the CDI-positive stools. Interestingly, both IL-8 and IL-23 were more frequently detected (88% and 90%, respectively) in the CDI-positive stools compared to the CDI-negative stools (54% and 68%, respectively) (Fig. 3).

To confirm the findings made from the data obtained from the Proteome Profiler Human Cytokine Array assay, fecal concentrations of the biomarkers whose band intensities demonstrated significant differences between the CDI-positive and CDI-negative stools were determined by quantitative ELISA (R&D Systems, Minneapolis, MN). Table 2 shows the range, mean, and median concentrations of these biomarkers (IL-8, IL-23, TNF-α, IFN-γ, and IL-13), which were measured from 50 CDI-positive, 50 CDI-negative, and 45 non-diarrhea volunteer stools. The average fecal concentration of IL-8 (318.2 pg/ml) was significantly higher (p< 0.05) in CDI-positive stools than that of the CDI-negative (84.7 pg/ml) and hospitalized controls without diarrhea (79.8 pg/ml). However, the fecal concentration of IL-23 was significantly lower (p< 0.05) in the CDI-positive stools (722 pg/ml) than that of the CDI-negative (946.7 pg/ml) and the hospitalized controls without diarrhea (1617 pg/ml). Interestingly, IL-23 was detected in a greater number of the CDI-positive stools compared to the CDI-negative stools, but its fecal concentrations were significantly lower in the CDI-positive samples. This is in contrast to IL-8, which was present in a larger number of the CDI-positive stools and at higher concentrations than the CDI-negative stools. These results suggest that IL-23 and IL-8 may be significant in the immunopathogenesis of CDI.
Th1- and Th2-associated cytokines were examined to assess the nature of cell-mediated response that occurs during CDI. Th1- and Th2 cytokines were detected in both CDI-positive and CDI-negative stools by the Proteome Profiler Human Cytokine Array assay. The relative amounts of four key Th1 (IFN-γ, IL-2, IL-12, and TNF-α) and five Th2 (IL-4, IL-5, IL-6, IL-10, IL-13) cytokines that were detected in both groups is shown in Figure 4A. The CDI-positive stools generally showed higher relative amounts of both Th1 and Th2 cytokines than the CDI-negative stools. However, the differences in the relative amounts were not significant (p> 0.05) from that of the CDI-negative stools, except TNF-α and IL-13, which were significantly higher in CDI-positive stools \([P= 0.0001 \text{ (for TNF-α)} \text{ and } P= 0.016 \text{ (for IL-13)}]\). These observations were confirmed when the actual concentrations of these cytokines in the stools were measured by quantitative ELISA. The average fecal concentrations of TNF-α (59.8 pg/ml), IFN-γ (69.7 pg/ml), and IL-13 (323.8 pg/ml) were higher in the CDI-positive stools compared to that of CDI-negative stools \([\text{TNF-α (31.2 pg/ml)}, \text{IFN-γ (48.3 pg/ml)}, \text{and IL-13 (141.1 pg/ml)}]\) and hospitalized controls without diarrhea \([\text{TNF-α (22.3 pg/ml)}, \text{IFN-γ (57.6 pg/ml)}, \text{and IL-13 (234.3 pg/ml)}]\) (Fig. 4B). These data suggest that the inflammatory response to CDI is complex and encompasses both Th1 and Th2 response.

Fecal concentrations of lactoferrin and calprotectin were determined to evaluate whether these innate inflammatory proteins play a role in CDI pathogenesis. As shown in Figure 5, the median lactoferrin concentration in the CDI-positive stools (31.4 μg/ml) was 5-fold higher than that of the CDI-negative stools (6.3 μg/ml) and about 6-fold
higher than the hospitalized controls without diarrhea (5.6 μg/ml). The median concentration of calprotectin in the CDI-positive stools (18 μg/ml) was 3-fold higher than that of the CDI-negative stools (6.5 μg/ml) and 2-fold higher than that of the hospitalized controls without diarrhea (8.7 μg/ml). The lactoferrin concentrations of 88% of the CDI-positive stools and 44% of the CDI-negative stools were higher than the average concentration of the hospitalized controls without diarrhea. Also, 80% of the CDI-positive stools and 30% of the CDI-negative stools had calprotectin concentrations higher than the average concentration of the hospitalized controls without diarrhea. As evidenced by the high levels of lactoferrin and calprotectin present in the CDI-positive stools, these data together suggest that majority of the cases of CDI have colonic inflammation, whereas a minority of the cases of antibiotic-associated diarrhea without CDI shows colonic inflammation.

**DISCUSSION**

Major gaps still remain in our understanding of the immunopathogenesis of CDI, despite its increasing prevalence rates and poor patient outcomes. The relative amounts of 36 major biomarkers were evaluated from stools collected from CDI and non-CDI patients. Initially, 50 CDI-positive and 50 CDI-negative stools obtained from hospitalized patients with antibiotic-associated diarrhea were assessed using the Proteome Profiler Human Cytokine Array assay. Fecal concentrations of the biomarkers that were found by the initial Proteome Profiler Human Cytokine Array assay to be significantly different between the CDI-positive and CDI-negative stools were measured by quantitative
ELISA for comparison with that of the hospitalized controls without diarrhea. We have provided data demonstrating that the immunopathogenesis of CDI is complex and elicits both Th1 and Th2 response, with an increased expression of pro-inflammatory proteins. IL-8 and IL-23 appeared to be important in the immunopathogenesis of CDI. These two cytokines were detected in the majority of the CDI-positive stools compared to the CDI-negative stools. The average concentration of IL-8 in the CDI-positive stools was significantly (p< 0.05) higher than that of the stools from CDI-negative and hospitalized controls without diarrhea. IL-8 is a chemoattractant involved in the recruitment of neutrophils to sites of infection and has been implicated to play a key role in the pathogenesis of CDI (16). Increased levels of IL-8 is associated with more severe forms of CDI (6). Moreover, a single nucleotide polymorphism (SNP) in the promoter region of the IL-8 gene that increases its expression is associated with susceptibility to CDI (17). These reports are consistent with our data showing high levels of IL-8 in the majority of CDI-positive stools compared to the stools from the CDI-negative and hospitalized controls without diarrhea.

Remarkably, the average concentration of IL-23 in the CDI-positive stools was lower than that of the CDI-negative and hospitalized controls without diarrhea, even though IL-23 was present in a higher number of the CDI-positive stools. IL-23 is produced by activated macrophages and dendritic cells and plays an important role in host defense against bacterial infections and development of chronic inflammation (18). During bacterial infection, antigen-stimulated dendritic cells and macrophages produce IL-23 that promotes the development of Th17 cells leading to enhanced priming of memory T
cells (18, 19). This results in induction and production of a variety of inflammatory mediators that triggers potent inflammatory responses. IL-23 also stimulates generation of pro-inflammatory cytokines such as IL-1, IL-6, IFN-γ, and TNF-α through its effects on dendritic cells and macrophages (20-22). Our data demonstrates for the first time that the average fecal concentration of IL-23 in the stools of CDI-positive patients was significantly (P<0.05) lower than that of hospitalized controls without diarrhea and CDI-negative patients. This suggests that the amount of IL-23 produced during CDI may be inadequate to sustain the cellular immunity conferred by this cytokine in promoting the induction and proliferation of effector memory T cells. Thus, decreased production of IL-23 may explain the lack of robust immunological response exhibited by a proportion of CDI patients and may also relate to recurrence. Perhaps, boosting the level of IL-23 may help activate the cellular immune response required for a robust response to CDI. Our data appears to contrast the findings of Buonomo et al, who recently reported an increased IL-23 in a small number of human colon biopsies from CDI patients and two murine CDI models (23). However, both studies identified IL-23 as important in CDI. The present study involved a larger number of patients with CDI of variable severity compared with the smaller human and animal studies reported by Buonomo et al. The patient populations between the two studies as well as the samples tested are different. Further studies are needed to confirm these findings such as larger patient populations from different locations to further evaluate the global role of IL-23 in the immunopathogenesis of CDI.
The average concentrations of lactoferrin and calprotectin in healthy adults range between 1.45-4.6 µg/ml (24, 25) and ≤ 10 µg/ml (26), respectively. This is consistent with the concentrations obtained from our study; 6.8+/0.85 µg/ml (for lactoferrin) and 10.2+/0.92 µg/ml (for calprotectin). Elevated levels of lactoferrin and calprotectin in stools is associated with colonic inflammation (27). Our data shows that 88% and 80% of the CDI-positive stools had average lactoferrin and calprotection concentrations, respectively, higher than the hospitalized controls without diarrhea. These results agree with that of Shastri et al. (28), in which 85.1% and 82.8% of CDI patients stools had higher levels of lactoferrin and calprotectin, respectively, than healthy adults. Lactoferrin and calprotectin serve as part of the innate inflammatory response and so their overexpression during CDI may provide insight into the extent of inflammation associated with this infection.

It is important to note that a proportion of CDI-positive stool samples had levels of either lactoferrin (12%) or calprotectin (20%) below that of the hospitalized controls without diarrhea. This underscores the complex nature of CDI immunopathogenesis, as evidenced by a wide range of clinical and symptomatic phenotypes observed in CDI patients (29). These subjects with relatively low lactoferrin and calprotection concentrations may represent patients diagnosed with CDI who are simply colonized by C. difficile with diarrhea due to another cause. The more sensitive methods of detecting the C. difficile toxins do not always differentiate between C. difficile carriage and C. difficile-associated diarrhea. Combining the presence of an inflammatory biomarker in
diarrheal stools together with a positive assay for *C. difficile* toxin may move us closer to a gold standard for CDI diagnosis.

The mechanisms and factors that contribute to antibiotic-associated diarrhea may also trigger inflammation in patients that tested negative for CDI. In fact, our study shows that 44% and 30% of the CDI-negative stools had average lactoferrin and calprotectin concentrations, respectively, higher than that of the hospitalized controls without diarrhea. This offers insight into the inflammatory nature of a subset of patients with non-CDI hospital-associated diarrhea. Some of the pro-inflammatory cytokines and inflammatory-associated proteins that were found in the CDI-positive stools were also detected in the CDI-negative stools. As a result, confirmation of the extent of colonic inflammation by testing colonic mucosal tissues would have been useful, although that is beyond the scope of this study.

We also note that secretory forms of diarrhea can theoretically dilute intra-luminal contents. If the concentration of mucosal cytokines was the same in patients with secretory diarrhea as normal controls, the patients with diarrhea are likely to have lower levels of detectable fecal cytokines. This could be a factor in the study. However, we also compared cytokine levels in CDI-negative patients with diarrhea. Theoretically, fecal cytokines from both diarrheal groups should be diluted, but some of the cytokines detected such as IL-8 were higher in CDI-positive diarrhea stools when compared to the non-diarrheal stools.
The differentiation of naïve CD4\(^+\) helper T-cells into either Th1 or Th2 cells is critical in the development of adaptive immune response (30). Th1 inflammatory response usually induces IFN-\(\gamma\) production leading to activation of phagocytes, whereas Th2 response results in humoral immunity, allergic inflammation, and stimulates host resistance to intracellular infections or agents (31, 32). The patterns of Th1- and Th2-associated cytokines found in the CDI-positive stools were not distinct from that of the CDI-negative stools. However, the concentrations of TNF-\(\alpha\) and IL-13 in the stools of CDI-positive patients were significantly higher than that of the CDI-negative and hospitalized controls without diarrhea. This suggests a mixed Th1/Th2 response during CDI and infers that the host response to CDI is complex, pro-inflammatory, and encompasses both the innate and the adaptive arms of the immune system. These results may also denote a probable intracellular response to the toxins and an extracellular response to the bacterium.
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engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13:715-725.


FIGURE LEGENDS

Figure 1. Different inflammatory biomarkers detected in the clinical stool samples examined. Stools (300 mg) from 100 antibiotic-associated diarrheal patients (50 CDI-positive and 50 CDI-negative) were evaluated for the presence of 36 inflammatory-associated proteins using the Proteome Profiler Human Cytokine Array Panel A Kit (R&D Systems, Minneapolis, MN). Key: Pos. Ctl, positive control; Neg. Ctl, negative control; CD40L, CD40 ligand; IFN-γ, interferon-gamma, IL-1α, interleukin-1 alpha; IL-1β, interleukin-1 beta; IL-1ra, interleukin-1 receptor antagonist; IL-8, interleukin-8, IL-23; interleukin-23; MIF, macrophage migration inhibitory factor; PAI-1, plasminogen activator inhibitor-1.

Figure 2. Fold change in amount of inflammatory markers detected in CDI-positive and CDI-negative stools. Stools (300 mg) from 100 antibiotic-associated diarrheal patients (50 CDI-positive and 50 CDI-negative) were evaluated for the presence of 36 inflammatory biomarkers using the Proteome Profiler Human Cytokine Array Panel A kit (R&D Systems, Minneapolis, MN). Band intensities were determined and converted into pixel densities using ImageJ (National Institutes of Health, Bethesda, MD). Error bars represent the standard deviations from the mean values for each inflammatory biomarker. Significant differences between CDI-positive and CDI-negative stools are denoted by “**”. The p-values based on non-parametric t-test are C5a (0.011), CD40L (0.042), G-CSF (0.045), I309 (0.030), IL-13 (0.001), IL-16 (0.031), IL-27 (0.027), MCP-1 (0.013), TNF-α (0.021), IL-8 (0.004), IL-23 (0.002).
Figure 3. IL-8 and IL-23 were present in the majority of CDI-positive and CDI-negative stools. A. IL-8 and IL-23 were detected in a greater number of CDI-positive stools than CDI-negative stools. Stools (300 mg) from 100 antibiotic-associated diarrheal patients (50 CDI-positive and 50 CDI-negative) were evaluated for the presence of 36 inflammatory-associated proteins using the Proteome Profiler Human Cytokine Array Panel A Kit (R&D Systems, Minneapolis, MN). Stools from the hospitalized controls without diarrhea were not evaluated by this initial assay, but were included retrospectively in the quantitative ELISA for comparison.

B. CDI-positive stools contain high amounts of IL-8 and relatively low amounts of IL-23 compared to stools from CDI-negative diarrheic patients and hospitalized controls without diarrhea. The concentrations of IL-8 and IL-23 were determined in stools (50 CDI-positive, 50 CDI-negative, and 45 non-diarrhea controls) by quantitative ELISA (R&D Systems, Minneapolis, MN). Mann-Whitney two-tailed test showed significant difference between CDI-positive vs non-diarrhea control (p= 0.0001); CDI-positive vs CDI-negative diarrhea (P = 0.002); and CDI-negative vs non-diarrhea controls (p= 0.006) for IL-8 and CDI-positive vs non-diarrhea control (p= 0.001); CDI-positive vs CDI-negative diarrhea (P = 0.003); and CDI-negative vs non-diarrhea controls (p= 0.0001) for IL-23. The horizontal bar = mean concentrations in pg/ml.

Figure 4. Comparison of Th1 and Th2 cytokines in CDI-positive and CDI-negative stools. A. Fold change in amount of Th1 (IFN-γ, IL-2, IL-12, TNF-α) and Th2- (IL-4, IL-5, IL-6, IL-10, IL-13) cytokines obtained from the initial Proteome Profiler Human Cytokine Array Panel A Kit (R&D Systems, Minneapolis, MN).
Cytokine Array assay. Stools (300 mg) from 100 antibiotic-associated diarrheal patients (50 CDI-positive and 50 CDI-negative) were evaluated for the presence of 36 inflammatory proteins using the Proteome Profiler Human Cytokine Array Panel A kit (R&D Systems, Minneapolis, MN). Data expressed as the mean of the relative band intensity of each cytokine. Stools from the hospitalized controls without diarrhea were not evaluated by this initial assay, but were included retrospectively in the quantitative ELISA for comparison. Error bars represent the standard error of measurement between two replicates per sample. * = P < 0.05.

B. Concentrations of IFN-γ, TNF-α, and IL-13 in CDI-positive stools and stools from CDI-negative diarrheic patients and hospitalized controls without diarrhea, determined by quantitative ELISA (R&D Systems, Minneapolis, MN). The Krustal-Wallis test showed significant differences between the means (p< 0.0001). Horizontal bar = mean concentration in µg/ml. Key: IFN-γ, interferon-gamma; IL-2, interleukin-2; IL-12, interleukin-12; TNF-α, tumor necrosis factor-alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-10, interleukin-10; IL-13, interleukin-13.

Figure 5. CDI-positive stools contain high amounts of lactoferrin and calprotectin than CDI-negative diarrheic stools and hospitalized controls without diarrhea. Concentrations of lactoferrin and calprotectin were measured in stools from 50 CDI-positive and CDI-negative patients and hospitalized controls without diarrhea. Fecal lactoferrin and calprotectin concentrations were determined using the IBD-SCAN (Techlab, Blacksburg, VA) and the HK325 Human Calprotectin ELISA kit (Hycult Biotech, Plymouth Meeting, PA), respectively. Median lactoferrin concentrations were
31.4 µg/ml (CDI-positive), 6.3 µg/ml (CDI-negative), and 5.6 µg/ml (hospitalized controls without diarrhea). Median calprotectin concentrations were 18.0 µg/ml (CDI-positive), 6.5 µg/ml (CDI-negative), and 8.7 µg/ml (hospitalized controls without diarrhea). One-way analysis of variance showed significant differences between the means of the CDI-positive, CDI-negative, and non-diarrhea controls in both lactoferrin and calprotectin (P < 0.0001).

**TABLES**

**Table 1.** Characteristics of study population.

**Table 2.** Fecal concentrations of IL-13, IL-8, IL-23, TNF-α, and IFN-γ (in pg/ml), lactoferrin and calprotectin (in µg/ml) determined by quantitative ELISA (R&D Systems, Minneapolis, MN) from the CDI-positive diarrheal, CDI-negative diarrheal, and non-diarrheal stools evaluated.
### Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CDI-Positive (n=50)</th>
<th>CDI-Negative (n=50)</th>
<th>Control Patients with no Diarrhea (n=45)</th>
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<tbody>
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<td>Average age (range)</td>
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<td>8</td>
<td>11</td>
</tr>
<tr>
<td>H/L</td>
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<td>6</td>
<td>9</td>
</tr>
<tr>
<td>W/C</td>
<td>28</td>
<td>29</td>
<td>25</td>
</tr>
<tr>
<td>Other</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>27</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>26</td>
<td>26</td>
</tr>
</tbody>
</table>

CDI = Patients with *Clostridium difficile* infection, B/AA, Black/African-American; H/L, Hispanic/Latino; W/C, White/Caucasian.
Figure 1
Figure 2

[Bar chart showing fold change in amount of inflammatory markers for CDI-negative diarrhea and CDI-positive diarrhea.]

Fold change in amount of inflammatory markers

CDI-NEGATIVE DIARRHEA

CDI-POSITIVE DIARRHEA

Markers include IL-1α, IL-1β, MIP-1α, MIP-1β, GM-CSF, IL-6, IL-8, GRO-α, KC, MCP-1, and others.
Figure 3

A

Total number of IL-23-positive donors

CDI-NEGATIVE DIARRHEA
CDI-POSITIVE DIARRHEA

IL-8
IL-23

B

IL-8

Concentration in stools (ug/ml)

Hospitalized Controls Without Diarrhea
CDI-Negative Diarrhea
CDI-Positive Diarrhea

IL-23

Concentration in stools (ug/ml)

Hospitalized Controls Without Diarrhea
CDI-Negative Diarrhea
CDI-Positive Diarrhea
<table>
<thead>
<tr>
<th>Inflammatory Markers</th>
<th>CDI-Positive N=50</th>
<th>CDI-Negative N=50</th>
<th>Non-Diarrhea Control N=45</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>IL-13</td>
<td>323.8</td>
<td>277.4</td>
<td>1150</td>
</tr>
<tr>
<td>IL-8</td>
<td>318.2</td>
<td>242.0</td>
<td>1150</td>
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<tr>
<td>IL-23</td>
<td>722.0</td>
<td>380.5</td>
<td>7069</td>
</tr>
<tr>
<td>TNF-α</td>
<td>59.8</td>
<td>33.7</td>
<td>163.6</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>69.7</td>
<td>58.0</td>
<td>229.7</td>
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<tr>
<td>Lactoferrin</td>
<td>43.4</td>
<td>31.4</td>
<td>155.2</td>
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<td>Calprotectin</td>
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<td>18.0</td>
<td>2.8-70.2</td>
</tr>
<tr>
<td>Characteristics</td>
<td>CDI-Positive (n= 50)</td>
<td>CDI-Negative (n= 50)</td>
<td>Control Patients with no Diarrhea (n= 45)</td>
</tr>
<tr>
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<td>---------------------</td>
<td>---------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Average age (range)</td>
<td>58 (2-91)</td>
<td>56 (21-83)</td>
<td>63 (22-85)</td>
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<tr>
<td>Ethnicity</td>
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<tr>
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</tr>
<tr>
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</table>

CDI = Patients with *C. difficile* infection, B/AA, Black/African-American; H/L, Hispanic/Latino; W/C, White/Caucasian.
<table>
<thead>
<tr>
<th>INFLAMMATORY MARKERS</th>
<th>CDI-POSITIVE N= 50</th>
<th>CDI-NEGATIVE N= 50</th>
<th>NON-DIARRHEA CONTROL N= 45</th>
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<td>RANGE</td>
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<td>35.7-1150</td>
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<tr>
<td>IFN-γ</td>
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<td>LACTOFERRIN</td>
<td>43.4</td>
<td>31.4</td>
<td>3.0-155.2</td>
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<td>2.8-70.2</td>
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