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Aging impairs protective host defenses against *Clostridioides* (*Clostridium*) *difficile* infection in mice by suppressing neutrophil and IL-22 mediated immunity

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Abstract

Background.—Morbidity and mortality associated with *Clostridioides* (formerly *Clostridium*) *difficile* infection (CDI) rises progressively with advanced age (> 65 years) due in part to perturbations of the gut microbiota and immune dysfunction. Epidemiological data of community-acquired CDI suggests increased susceptibility may begin earlier during middle-age (45–64 years) but the causation remains unknown.

Methods.—Middle-aged (12–14 months) and young (2–4 months) adult mice were infected with *C. difficile*, and disease severity, gut microbiome and innate immune response were compared. Cytokine reconstitution studies were performed in infected middle-aged mice.

Results.—Infection of middle-aged mice with *C. difficile* led to greater disease compared to young controls, which was associated with increases in *C. difficile* burden and toxin titers, and elevated bacterial translocation. With the exception of an expansion of *C. difficile* in middle-aged mice, microbiome analysis revealed no age-related differences. In contrast, middle-aged mice displayed a significant defect in neutrophil recruitment to the colon, with diminished levels of

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innate immune cytokines IL-6, IL-23 and IL-22. Importantly, recombinant IL-22 administration during CDI reduced morbidity and prevented death in middle-aged mice.

Conclusion.—Increased susceptibility to *C. difficile* occurs in middle-aged mice modeling the community-acquired CDI demographics and is driven by an impaired innate immune response.

Keywords

Clostridioides difficile, *C. difficile*; aging; middle-age; neutrophils; IL-22

Background

Clostridioides (formerly *Clostridium*) *difficile* infection (CDI), the leading cause of antibiotic-associated diarrhea in industrialized countries, is an urgent threat to public health. In the U.S., >300,000 healthcare-acquired infections occur annually, accounting for \$8.2 billion in health care expenses [1]. Age is a major risk factor for CDI, with the majority of healthcare-acquired infections occurring in the elderly (> 65 years of age) [2]. CDI in this population often results in severe disease with adverse consequences due to comorbidity, including increased risk for disease recurrence and death [2]. Advanced age is also associated with community-acquired CDI, although studies have consistently shown that patients with community-acquired CDI are younger than those with healthcare-acquired infections (median age ~45 vs 65, respectively) [3]. The reasons for the age-related differences between community- and healthcare-acquired CDI are poorly defined.

With aging, physiological changes occur that result in immune dysfunction [4]. Some of the changes are progressive (e.g. decline in T-cell function and production of antibodies), whereas other changes are only found with advanced age (> 65 years (e.g. diminished macrophage responses) [5, 6]. Emerging evidence highlights that aging promotes onset of tissue-specific low grade inflammation which diminishes the ability of the host to mount effective innate immune responses against infection. Advanced age also reduces the expression of microbial pattern recognition receptors, alters the recruitment and function of innate immune cells, and is associated with elevated basal inflammation and secretion of proinflammatory cytokines, including IL-1 β , IL-6 and TNF- α [7]. Several recent studies have demonstrated impaired innate and humoral responses to CDI in mice of advanced age (> 18 months) [8, 9], which may explain the increased morbidity and mortality to CDI in elderly patients.

Innate immunity is essential for host survival following CDI [10]. Studies have shown innate immune sensors Nod1 and TLR4, and its adapter protein MyD88, are required for controlling *C. difficile* spore production and preventing death induced by the systemic spread of pathobionts, commensal bacteria that can breach the intestinal epithelial barrier [11–14]. Increased bacterial translocation and death have also been reported in mice with CDI that have undergone neutrophil-depletion [11, 12, 14, 15]. In response to CDI, neutrophils accumulate in areas where toxin has damaged the epithelium to create a barrier that prevents translocation of pathobionts into the underlying tissues [14]. Pathobionts that reach the systemic circulation are quickly eliminated through complement-mediated killing by neutrophils [16]. This critical process is regulated by the cytokine IL-22, which in

addition to containing the spread of commensal bacteria [17] also promotes expression of antimicrobial peptides and other factors important for ensuring spatial separation between commensal bacteria and the epithelial barrier, and shaping the composition of the intestinal microbiome [18].

Intestinal microbiota are critical determinants of CDI susceptibility and disease outcome [19]. During the aging process, significant shifts in the composition of the microbiota occur, which are associated with a loss in species diversity and immune dysfunction [20]. However, it remains unclear at what age the shifts begin and whether they are the effect of changes in environmental conditions or physiological processes. Recently, age-related alterations in the microbiota were found to underlie deficiencies in innate immunity against CDI in elderly mice (> 18 months), which were reversed upon transfer of “youthful” gut microbiota prior to infection [9], suggesting that the microbiota is responsible for age-related immunodeficiency. The involvement of IL-22 in this process remains unknown. The present studies were conducted to evaluate whether increased susceptibility to CDI occurs at an earlier age, and determine the impact of IL-22 on CDI disease outcome in middle-age hosts modeling community-acquired disease onset.

METHODS

Mice and infection protocol.

Young (1.5–2 months) and middle-aged (12–14 months) C57BL/6 mice (Jackson Laboratory) of both sexes were used in the experiments. All mice were bred and aged under pathogen-free sterile conditions at the University of Texas Medical Branch (UTMB). Using methods established by Chen et al [21], mice received an antibiotic cocktail in their drinking water for 3 days and were returned to fresh water for 2 days before receiving a single dose of clindamycin (10 mg/kg, i.p.). Twenty-four hours later, mice were infected with *C. difficile* (10^3 spores/mouse, strain VPI 10463). Mice were monitored daily for weight loss, hunched posture, lethargy, stool consistency, occult blood, and rectal bleeding. All animal studies were approved by the Institutional Animal care and Use Committee of UTMB.

Bacterial quantification.

Bacterial burdens were determined by CFU assays. Fecal pellets were collected from individual mice, weighed and homogenized in sterile PBS. Following an incubation at 65°C for 30 min, homogenates were serially diluted and plated onto selective *C. difficile* agar (bioWORLD, Dublin, OH), supplemented with taurocholate, cycloserine-cefoxitin and defibrinated horse blood. Plates were placed at 37°C for 48 hrs under anaerobic conditions. To assess bacterial translocation, organs were homogenized in PBS, and serial dilutions plated on BHI agar. Following incubation at 37°C for 48 hrs under aerobic conditions, CFU were enumerated by manual counting.

Cytotoxicity assay.

CHO-K1 cells (ATCC CCL-61) were grown in 96-well plates (10^5 cells/well) in F12 medium with 10% FBS. Fecal pellets were homogenized in PBS (10 mg/mL) and centrifuged at $20,000 \times g$ for 20 min. The supernatants were serially diluted and added to the

cells. Plates were incubated at 37°C in 5% CO₂ for 24 hrs. Each plate included control wells untreated and treated with purified *C. difficile* toxin B (List Laboratories, Campbell, CA) and antitoxin (TechLab, Blacksburg, VA). The cytotoxicity titer was expressed as the reciprocal of the highest dilution that caused more than 80% cell rounding per gram of feces.

Histological analysis.

At select times, ceca and colons were harvested and the lengths recorded. The organs were opened longitudinally and Swiss rolled prior to fixation for 48 hrs in 10% formalin. Following paraffin embedding, 5 µm-sections were prepared and stained with hematoxylin and eosin (H&E). Histological scores (range 0–10) were obtained in a blinded fashion by two observers using the following parameters: a. Epithelial erosion (0, absent; 1, loss limited to the apical surface; 2, loss limited to the surface and upper halves of crypts; 3, loss extending down the entire length of the crypts; 4, crypt ablation; b. Inflammatory cell infiltration of mucosa and submucosa (0, absent; 1, mild inflammation; 2, moderate infiltration; 3, severe infiltration); c. Edema (0, absent; 1, mild/moderate; 2, Severe); d. Congestion (0, absent; 1, present). The overall score was the sum of each component score. For each section, quantification was performed on the two most affected areas at least 15 crypts apart, and the scores were averaged. Neutrophil numbers were quantified per 15 crypts for 4 noncontiguous areas at high power magnification (100x), and the scores were averaged.

16S rRNA sequencing and analysis.

Total DNA was extracted from frozen colon content specimens using the PowerSoil extraction kit (MO BIO Laboratories, Carlsbad, CA). Amplification and sequencing of the V1V3 region of the 16S rRNA gene was performed using the NEXTflex™ 16S V1V3 Amplicon-Seq Kit 2.0 (Bio Scientific, Austin, TX), and sequences were generated on the Illumina MiSeq platform (Illumina, San Diego, CA) with a minimum of 15,000 sequences generated per sample. Following 16S rRNA sequence generation, raw reads were filtered for high quality using the Lotus pipeline [22] followed by de novo clustering to operational taxonomic units (OTUs) at 97% sequence identity with UPARSE [23]. Bacterial community diversity and composition was evaluated using the QIIME v1.8 sequence analysis platform [24]. Taxonomy assignment of the representative sequence for each OTU was completed using the RDP classifier algorithm and the SILVA reference database (v123) [25]. Libraries were randomly subsampled to a depth of 16,300 sequences prior to the calculation of diversity metrics or assessment of differences in taxon relative abundance. Significant differences in alpha diversity and OTU abundances were assessed using the Mann-Whitney U test. Jaccard dissimilarities and stable non-metric multidimensional scaling (NMDS) coordinates were determined from untransformed abundance data using QIIME v1.8 and plotted in GraphPad PRISM® (v5.04). Significance of changes in community structure between groups was evaluated with Analysis of Similarities (ANOSIM) calculated using mothur v1.39.5 [26]. In addition to SILVA classification outlined above, specific representative OTU sequences of interest were analyzed by blastn against the NCBI 16S Microbial database for the top hit. Sequences have been deposited with the NCBI Short Read Archive under Bioproject number (in progress).

Analysis of mRNA expression.

Total RNA was extracted from whole colon tissue with the RNeasy Mini Kit (Qiagen). The RNA was reversely transcribed using iScript™ Reverse Transcription Supermix (Bio-Rad), and the complementary DNA used to perform quantitative PCR using iTaq™ Universal SYBR Green Supermix (Bio-Rad) on a ViiA™ 7 system (Applied Biosystems, Carlsbad, CA). Primers and expected PCR product sizes are shown in Table 1. Relative changes in target mRNA levels were calculated by the 2^{-CT} method with GAPDH mRNA as the reference standard.

ELISA assays.

Colon tissue was harvested, snap-frozen, and stored at -80°C until use. The tissue was weighed and homogenized in PBS containing 1% Triton X-100 and Complete Protease Inhibitor (Roche). Following centrifugation, supernatants were collected and assayed for cytokine levels using ELISA kits purchased from eBioscience. Results were normalized to total protein as determined by BCA assay (Bio-Rad).

In vivo administration of recombinant IL-22 (rIL-22).

Middle-aged mice were treated i.p. with 1 μg rIL-22 (eBioscience) or PBS 12 h after infection.

Statistical analysis.

Statistical analysis was performed by using Sigma Plot 12 (Systat Software Inc.). To compare bacterial loads, CFU were $\log_{10}$ transformed, and mean and standard error (SE) calculated. Differences in counts were evaluated by the Mann-Whitney rank sum test, disease-free survival data were examined by LogRank Survival analysis, and all other comparisons between groups were evaluated by Student's t test. A p -value < 0.05 was considered significant.

RESULTS

Middle-aged mice demonstrate increased sensitivity to *C. difficile* infection

Previous studies have demonstrated that male mice of advanced age (>18 months) are more susceptible to severe disease caused by CDI [8], in part because of gut microbiota-dependent alterations in protective innate host defenses [9]. However, it is unclear how and when the gut microbiota composition shifts from a resistant adult/middle-aged stage (45–65 years) to a more susceptible stage associated with extreme age (>65 years). Epidemiological evidence suggests that the decline in host resistance to CDI occurs earlier during middle-age, when physiological functions and metabolic responses are still preserved yet the microbiota has undergone a major shift [27]. To assess whether middle-aged mice have increased sensitivity to CDI, we inoculated young adult (1.5–2 months) and middle-aged (12–14 months) C57BL/6 mice with *C. difficile* spores by oral gavage. Beginning 3 days after infection, middle-aged mice lost significantly more weight than young control mice and continued to lose weight throughout the course of infection (Fig. 1A). Middle-aged mice also exhibited more clinical signs of disease, including weight loss ($>20\%$), diarrhea and hunched posture,

and were more likely to succumb to infection (Fig. 1B; mortality: 37.5% in middle-aged mice vs 12.5% in young mice).

We next sought to examine sex differences in susceptibility to CDI. Significant differences in weight loss between middle-aged and young male mice were not observed until 6 days after infection (Fig. 1C); whereas in middle-aged females, significant reductions in weight loss were evident after 3 days (Fig. 1C). Moreover, ~70% of middle-aged male mice developed symptoms during the course of infection, while only 17% of the young male mice exhibited signs of disease (Fig. 1D). In contrast, disease accelerated in aged female mice which showed disease symptoms as early as day 2 and to a greater extent than young female mice (83% of aged females developed symptoms and/or succumbed to infection vs. 10% of young female controls; Fig. 1D). Despite these observational differences, no statistically significant sex-dependent differences in weight loss or disease-free survival were observed between aged males and females, and thus data was combined for further analyses.

Bacterial clearance is impaired in middle-aged mice

For clues about the underlying cause of increased sensitivity to CDI, we quantified bacterial numbers and toxin titers in the stool of young and middle-aged mice. *C. difficile* burden was greater in middle-aged mice one week after infection, with ~10,000-fold higher bacterial numbers (Fig. 2A left). Similarly, cytotoxic analysis revealed significant increases in *C. difficile* toxin production in middle-aged mice compared to young controls (Fig. 2A right). Furthermore, middle-aged mice had significantly higher bacterial numbers in the MLN, spleen, liver and kidneys at necropsy on day 9 (Fig. 2B). Taken together, the CFU data suggest that changes occur during middle age that impair clearance of *C. difficile* and its enterotoxins in the colon, and containment of commensal bacteria that translocate across epithelial barrier.

Increased susceptibility of middle-aged mice to CDI is independent of the microbiome

To examine the effects of aging on microbial community structure and composition in *C. difficile* infected mice, we sequenced the V1V3 variable region of 16S rRNA genes from DNA isolated from frozen cecal contents. As seen in humans [27], alpha diversity of the microbiota was significantly increased in untreated, middle-aged mice compared with young controls (Fig. 3A). Following antibiotic exposure, diversity decreased dramatically with a ~80% loss ($p = 0.005$, Mann-Whitney U-test) in the number of operational taxonomic units (OTUs, clustered at 97% sequence identity) in both age groups, which remained lost following infection (Fig. 3A).

Next we assessed microbiota composition to identify signatures that may be increasing disease sensitivity in middle-aged individuals. As expected, given prior characterizations of the mouse gut microbiome [28], all untreated mice were dominated by Bacteroidetes, Firmicutes, and Verrucomicrobia phyla (Fig. 3B), which did not stratify by age. Antibiotic treatment led to an expected shift in the community structure with a significant reduction in Bacteroidetes and expansion of Proteobacteria, particularly in middle age mice in which both Enterococcaceae and Pseudomonadaceae increased. Infection restored dominance by Bacteroidetes, Firmicutes, Verrucomicrobia in both age groups. The most striking difference

between middle-aged and young infected mice was the increased abundance of OUT_11, classified in the Peptostreptococcaceae family, present in the older animals that was not measured in the younger CDI controls (Fig. 3C, mean count 2455 ± 1230 v 0 , $p = 0.045$). The representative sequence of OTU_11 demonstrated 99% identity to *C. difficile* by blast analysis against the NCBI 16S rRNA gene database. The presence of OTU_11 in middle-aged mice supports the increased *C. difficile* burden and stool cytotoxicity in middle-aged CDI mice (Fig. 2A).

Overall changes in composition were assessed using the abundance-adjusted Jaccard similarity index and visualized through non-metric multidimensional scaling (NMDS, Fig. 3D). Significance testing of differences in community structure (using Analysis of Similarities (ANOSIM)) demonstrated that communities present prior to antibiotic treatment were significantly different than those present following antibiotic treatment for young ($R = 0.783$, $p = 0.003$) and middle-aged ($R = 0.429$, $p = 0.004$) mice. Additionally, CDI resulted in a shift in community structure compared to antibiotic treated mice for both young ($R = 0.746$, $p < 0.001$) and the middle-aged ($R = 0.662$, $p = 0.001$) animals. However, the compositional shifts observed were independent of age or sex, as there were no significant differences in the overall microbiota composition between young and middle-aged mice, or males and females (Fig. 3D).

Neutrophil recruitment in response to *C. difficile* infection is diminished in middle-aged mice

The inability of middle-aged mice to control bacterial burden prompted us to examine the expression of IL-6 and IL-23, pleiotropic cytokines involved in the regulation of inflammation and immunity. Following *C. difficile* infection, IL-6 and IL-23 gene expression and protein levels in the colon were unchanged in middle-aged mice (Fig. 4A&B). In contrast, young controls showed significant cytokine induction and production after infection. Histological analysis revealed fewer infiltrating neutrophils into the lamina propria of middle-aged mice compared to young mice (Fig. 4C), which was paralleled by no changes in the induction of neutrophil markers CXCR2 and MPO (Fig. 4D). Middle-aged mice did show an induction of neutrophil CXCL2, although the increase was significantly lower compared with young mice (Fig. 4D). Taken together, the data suggest diminished neutrophil recruitment in middle-aged mice, which was associated with attenuated IL-6 and IL-23 responses.

Recombinant IL-22 (rIL-22) prevents lethality of *C. difficile* in middle-aged mice

Neutrophils and other innate immune cells are major producers of IL-22, a key cytokine associated with survival following *C. difficile* infection [29]. Consistent with a dampened innate response, we found that IL-22 mRNA expression and protein were significantly decreased in middle aged mice after CDI (Fig. 5A). To determine if IL-22 affords protection in older subjects, we treated middle-aged CDI mice with rIL-22 or vehicle control. As observed in younger mice [16], rIL-22 rescued aged mice from clinical disease and mortality (Fig. 5B). Importantly, rIL-22 significantly reduced the amount of inflammation induced in the colon compared with vehicle controls (Fig. 5C&D), and reduced the numbers of bacteria translocating to the MLN by >100 fold (log CFU = 4.2 ± 1.0 in rIL-22 treated mice vs. 6.5

± 1.2 in vehicle control mice; $p < 0.02$), indicating restoration of host protective immunity. Interestingly, rIL-22 increased expression levels of *Il22* and its down-stream target genes involved in barrier protection (including *Reg3g*, *Saa1*, *Muc2* and *Muc3*), and promoted recruitment and activation of neutrophils, as indicated by the increased expression levels of *Cxcr2* and *Mpo* (Fig. 5E). These results demonstrate that aging impairs the IL-22 response to CDI, and that the cytokine is a major mediator of protection against toxin-induced damage and disease.

DISCUSSION

Aging is an intricate process that has disruptive effects on many host defense systems, including the microbiota and immune systems [4, 7, 27]. Although advanced age is a well-established risk factor for symptomatic CDI and infection-related mortality [30], efforts to elucidate the mechanisms involved in age-associated risk have only recently begun [8, 9]. Our studies extend the findings of these recent reports and demonstrate increased sensitivity to CDI begins prior to the onset of advanced age, at a time when physiological functions, metabolic responses, and microbiota community structures are still relatively preserved rather than in decline [27, 31]. Although there have been very few studies, alterations in innate immune function, which led to increased sensitivity to infection and immune insult, have been reported in middle-aged hosts [32, 33]. In support of this, we found that impaired neutrophil recruitment and suppression of IL-22 lead to heightened CDI mortality in this understudied age group. Together, these data provide further evidence that innate immune pathways play a critical role in mucosal defense against CDI in aging hosts.

Aging had a dramatic impact on bacterial colonization and toxin production in the large intestine and dissemination of bacteria to extra-intestinal organs one week after CDI, suggesting that innate defenses, such as neutrophil-mediated responses, which have been shown to be critical for controlling infection [14, 34], are altered in middle-aged hosts. Indeed, neutrophil recruitment was diminished in infected middle-aged mice. Although the underlying cause is not known, age-related aberrations in the early synthesis and secretion of inflammatory mediators, may account for the defective neutrophil response [35–37]. Consistent with this, we found an age-dependent reduction of IL-23 in response to CDI. In murine models of *C. difficile*, interventions to abrogate IL-23, through genetic deficiency or antibody-mediated neutralization, reduce levels of neutrophil recruiting chemokines (e.g. CXCL1 and CXCL2), which significantly diminishes neutrophil infiltration to the colon [38–40]. Although, the relationship between aging and IL-23 expression remains to be firmly established, studies using bone marrow-derived dendritic cells, which produce IL-23 in response to *C. difficile* toxin exposure [41], have reported that IL-23 secretion is increased in TLR-stimulated DCs from elderly mice compared to young controls [42]. By contrast, TLR function in DCs from the spleen and lymph nodes are unperturbed [43], suggesting that the effects of aging on DC responses are complex and reflective of many factors, including cell-type and tissue conditions. Studies investigating age-associated effects on neutrophil functions have shown both unaltered and impaired abilities in chemokine secretion and killing of phagocytosed pathogens [44], thus it remains to be determined what affect aging has on neutrophil function in the intestine in responses to enteric infections.

The intestinal microbiota has a significant influence on CDI susceptibility and disease outcome. Previous studies have shown the composition of the microbiota changes with age, with significant differences observed between children, adults and the elderly [27, 45]. Our results are in agreement with those of recent studies indicating the compositions are similar between young and middle-age adults [27]. In contrast to elderly hosts [9], we show that the age-related difference in severity observed in middle-aged mice is unlikely the result of differences in the microbiota, as the composition remained similar following broad-spectrum antibiotic exposure and subsequent infection with *C. difficile*, as shown by β -diversity analysis in Fig. 3C. These results suggest the microbiota is not the dominant modifier of CDI outcome in middle-age mice, and that increased sensitivity is driven primarily by an altered host response, such as early-onset innate immune defects. One possible explanation for the discrepancy between study results is that by rearing and aging mice in the same vivarium room we were able to control for microbiota differences to a degree not achieved in the previous study, and therefore were able to study the innate immune differences that come with aging independent of microbiota changes. Alternatively, and less likely, our 16S analysis missed small differences. By performing fecal microbiota transplants between young and middle-aged mice and looking for differences in disease outcome we would be able to confirm that the microbiota has little to no role in increasing susceptibility to *C. difficile* in middle-age hosts.

An important innate immune defense mechanism against CDI is IL-22 [16, 46]. Produced primarily by innate lymphoid cells and T lymphocytes, IL-22 expression is regulated by IL-23, IL-6, and IL-1 β to control intestinal microbiota [18]. In our study we found aging decreased expression of IL-23 and IL-6, and to a lesser extent IL-1 β (data not shown) in response to infection, which in turn was associated with diminished IL-22 production. The inability of middle-aged mice to mount a robust IL-22 response to CDI promoted mortality, as cytokine reconstitution led to improvements in both clinical disease and survival. Importantly, middle-aged mice treated with rIL-22 harbored fewer numbers of bacteria in the MLN compared to untreated controls. This observation is consistent with findings in IL-22-deficient mice that show the cytokine mediates protection by limiting dissemination of translocated bacteria following *C. difficile* toxin-induced intestinal damage, a protection process mediated through neutrophil-killing regulated by the complement system [16]. Although a deficiency in IL-22 alone was shown to have no impact on the extent of intestinal damage following CDI or recruitment of neutrophils to the site of infection in young adult mice [16, 46], we found that rIL-22 treatment in middle-aged mice reduced the amount of damage inflicted by the bacteria and restored neutrophil recruitment and activation, as indicated by increased expression of CXCR2 and MPO, respectively. During the aging process neutrophils develop phenotypic and functional abnormalities. They begin to produce lower levels of MPO and reactive oxygen species hindering their ability to kill pathogens, and develop additional impairments that affect cross-talk with other cells that results in poor neutrophil accumulation and resolution of inflammation [47]. Thus, it is plausible rIL-22 reverses neutrophil dysfunction through the promotion of other factors secreted by epithelial and other innate immune cells. This is consistent with a recent study showing that in response to chemical induced injury, intestinal epithelial cells secrete antimicrobial peptides to promote the recruitment of IL-22-producing neutrophils to restore

mucosal IL-22 levels [48]. Neutrophils are an important source of IL-22 in the intestine, as neutrophil depletion correlates with a reduction in the expression of IL-22 and antimicrobial peptides [49]. Additional studies involving the adoptive transfer of neutrophils from young to middle-aged mice are needed to determine whether neutrophil dysfunction plays a role in increased CDI susceptibility.

CONCLUSION

The observed age-related decline in innate immunity broadens our understanding of the effects of aging on host defenses against enteric infection. Importantly, we demonstrated aging suppresses the induction of several innate cytokines, including IL-23 and IL-22, during CDI that led to poor neutrophil recruitment to the large intestine and death associated with higher bacterial burden and toxin production and the accumulation of commensal bacteria in extra-intestinal tissues. The insights gained from these studies provide new mechanistic ideas about the pathophysiology and immunology of CDI in aging hosts, and identify a potential signaling pathway as a target for immune intervention to prevent pathology and increase survival in older individuals infected with *C. difficile*.

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REFERENCES

1. Zhang S, Palazuelos-Munoz S, Balsells EM, Nair H, Chit A, Kyaw MH. Cost of hospital management of Clostridium difficile infection in United States-a meta-analysis and modelling study. BMC infectious diseases 2016; 16:447. [PubMed: 27562241]
2. Donskey CJ. Clostridium difficile in Older Adults. Infectious disease clinics of North America 2017; 31:743–56. [PubMed: 29079158]
3. Gupta A, Khanna S. Community-acquired Clostridium difficile infection: an increasing public health threat. Infection and drug resistance 2014; 7:63–72. [PubMed: 24669194]
4. El Chakhtoura NG, Bonomo RA, Jump RLP. Influence of Aging and Environment on Presentation of Infection in Older Adults. Infectious disease clinics of North America 2017; 31:593–608. [PubMed: 29079150]
5. Wong C, Goldstein DR. Impact of aging on antigen presentation cell function of dendritic cells. Current opinion in immunology 2013; 25:535–41. [PubMed: 23806201]
6. Oishi Y, Manabe I. Macrophages in age-related chronic inflammatory diseases. NPJ aging and mechanisms of disease 2016; 2:16018. [PubMed: 28721272]
7. Bandaranayake T, Shaw AC. Host Resistance and Immune Aging. Clinics in geriatric medicine 2016; 32:415–32. [PubMed: 27394014]
8. van Opstal E, Kolling GL, Moore JH, 2nd, et al. Vancomycin Treatment Alters Humoral Immunity and Intestinal Microbiota in an Aged Mouse Model of Clostridium difficile Infection. The Journal of infectious diseases 2016; 214:130–9. [PubMed: 26917573]

9. Shin JH, Gao Y, Moore JH, 2nd, et al. Innate Immune Response and Outcome of Clostridium difficile Infection Are Dependent on Fecal Bacterial Composition in the Aged Host. *The Journal of infectious diseases* 2018; 217:188–97. [PubMed: 28968660]
10. Abt MC, McKenney PT, Pamer EG. Clostridium difficile colitis: pathogenesis and host defence. *Nature reviews Microbiology* 2016; 14:609–20. [PubMed: 27573580]
11. Hasegawa M, Yamazaki T, Kamada N, et al. Nucleotide-binding oligomerization domain 1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen. *J Immunol* 2011; 186:4872–80. [PubMed: 21411735]
12. Hasegawa M, Kamada N, Jiao Y, Liu MZ, Nunez G, Inohara N. Protective role of commensals against Clostridium difficile infection via an IL-1beta-mediated positive-feedback loop. *J Immunol* 2012; 189:3085–91. [PubMed: 22888139]
13. Ryan A, Lynch M, Smith SM, et al. A role for TLR4 in Clostridium difficile infection and the recognition of surface layer proteins. *PLoS pathogens* 2011; 7:e1002076. [PubMed: 21738466]
14. Jarchum I, Liu M, Shi C, Equinda M, Pamer EG. Critical role for MyD88-mediated neutrophil recruitment during Clostridium difficile colitis. *Infection and immunity* 2012; 80:2989–96. [PubMed: 22689818]
15. Ng J, Hirota SA, Gross O, et al. Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome. *Gastroenterology* 2010; 139:542–52, 52 e1–3. [PubMed: 20398664]
16. Hasegawa M, Yada S, Liu MZ, et al. Interleukin-22 regulates the complement system to promote resistance against pathobionts after pathogen-induced intestinal damage. *Immunity* 2014; 41:620–32. [PubMed: 25367575]
17. Sonnenberg GF, Monticelli LA, Alenghat T, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science* 2012; 336:1321–5. [PubMed: 22674331]
18. Schreiber F, Arasteh JM, Lawley TD. Pathogen Resistance Mediated by IL-22 Signaling at the Epithelial-Microbiota Interface. *Journal of molecular biology* 2015; 427:3676–82. [PubMed: 26497621]
19. Ross CL, Spinler JK, Savidge TC. Structural and functional changes within the gut microbiota and susceptibility to Clostridium difficile infection. *Anaerobe* 2016; 41:37–43. [PubMed: 27180006]
20. Biagi E, Candela M, Turroni S, Garagnani P, Franceschi C, Brigidi P. Ageing and gut microbes: perspectives for health maintenance and longevity. *Pharmacological research* 2013; 69:11–20. [PubMed: 23079287]
21. Chen X, Katchar K, Goldsmith JD, et al. A mouse model of Clostridium difficile-associated disease. *Gastroenterology* 2008; 135:1984–92. [PubMed: 18848941]
22. Hildebrand F, Tadeo R, Voigt AY, Bork P, Raes J. LotuS: an efficient and user-friendly OTU processing pipeline. *Microbiome* 2014; 2:30. [PubMed: 27367037]
23. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods* 2013; 10:996–8. [PubMed: 23955772]
24. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature methods* 2010; 7:335–6. [PubMed: 20383131]
25. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* 2013; 41:D590–6. [PubMed: 23193283]
26. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and environmental microbiology* 2009; 75:7537–41. [PubMed: 19801464]
27. Odumaki T, Kato K, Sugahara H, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC microbiology* 2016; 16:90. [PubMed: 27220822]
28. Nguyen TL, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? *Disease models & mechanisms* 2015; 8:1–16. [PubMed: 25561744]

29. Zindl CL, Lai JF, Lee YK, et al. IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis. *Proceedings of the National Academy of Sciences of the United States of America* 2013; 110:12768–73. [PubMed: 23781104]
30. Jump RL. *Clostridium difficile* infection in older adults. *Aging health* 2013; 9:403–14. [PubMed: 24955106]
31. Maffei VJ, Kim S, Blanchard Et, et al. Biological Aging and the Human Gut Microbiota. *The journals of gerontology Series A, Biological sciences and medical sciences* 2017; 72:1474–82.
32. Kohman RA, Crowell B, Kusnecov AW. Differential sensitivity to endotoxin exposure in young and middle-age mice. *Brain, behavior, and immunity* 2010; 24:486–92.
33. Farazi M, Cohn Z, Nguyen J, Weinberg AD, Ruby CE. Early-onset age-related changes in dendritic cell subsets can impair antigen-specific T helper 1 (Th1) CD4 T cell priming. *Journal of leukocyte biology* 2014; 96:245–54. [PubMed: 24714553]
34. Jose S, Madan R. Neutrophil-mediated inflammation in the pathogenesis of *Clostridium difficile* infections. *Anaerobe* 2016; 41:85–90. [PubMed: 27063896]
35. Bruunsgaard H, Pedersen AN, Schroll M, Skinhoj P, Pedersen BK. Impaired production of proinflammatory cytokines in response to lipopolysaccharide (LPS) stimulation in elderly humans. *Clinical and experimental immunology* 1999; 118:235–41. [PubMed: 10540184]
36. Himi T, Yoshioka I, Kataura A. Influence of age on the production of interleukin-8-like chemokine (GRO/CINC-1) in rat nasal mucosa. *Eur Arch Otorhinolaryngol* 1997; 254:101–4. [PubMed: 9065665]
37. Hobden JA, Masinick SA, Barrett RP, Hazlett LD. Proinflammatory cytokine deficiency and pathogenesis of *Pseudomonas aeruginosa* keratitis in aged mice. *Infection and immunity* 1997; 65:2754–8. [PubMed: 9199446]
38. McDermott AJ, Frank CR, Falkowski NR, McDonald RA, Young VB, Huffnagle GB. Role of GM-CSF in the inflammatory cytokine network that regulates neutrophil influx into the colonic mucosa during *Clostridium difficile* infection in mice. *Gut microbes* 2014; 5:476–84. [PubMed: 25045999]
39. McDermott AJ, Falkowski NR, McDonald RA, Pandit CR, Young VB, Huffnagle GB. Interleukin-23 (IL-23), independent of IL-17 and IL-22, drives neutrophil recruitment and innate inflammation during *Clostridium difficile* colitis in mice. *Immunology* 2016; 147:114–24. [PubMed: 26455347]
40. Buonomo EL, Madan R, Pramoongjago P, Li L, Okusa MD, Petri WA, Jr. Role of interleukin 23 signaling in *Clostridium difficile* colitis. *The Journal of infectious diseases* 2013; 208:917–20. [PubMed: 23776194]
41. Cowardin CA, Kuehne SA, Buonomo EL, Marie CS, Minton NP, Petri WA, Jr. Inflammasome activation contributes to interleukin-23 production in response to *Clostridium difficile*. *mBio* 2015; 6.
42. El Mezayen R, El Gazzar M, Myer R, High KP. Aging-dependent upregulation of IL-23p19 gene expression in dendritic cells is associated with differential transcription factor binding and histone modifications. *Aging cell* 2009; 8:553–65. [PubMed: 19624579]
43. Tan SY, Cavanagh LL, d'Advigor W, Shackel N, Fazekas de St Groth B, Weninger W. Phenotype and functions of conventional dendritic cells are not compromised in aged mice. *Immunology and cell biology* 2012; 90:722–32. [PubMed: 22231652]
44. Drew W, Wilson DV, Sapey E. Inflammation and neutrophil immunosenescence in health and disease: Targeted treatments to improve clinical outcomes in the elderly. *Experimental gerontology* 2017.
45. Mariat D, Firmesse O, Levenez F, et al. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC microbiology* 2009; 9:123. [PubMed: 19508720]
46. Sadighi Akha AA, McDermott AJ, Theriot CM, et al. Interleukin-22 and CD160 play additive roles in the host mucosal response to *Clostridium difficile* infection in mice. *Immunology* 2015; 144:587–97. [PubMed: 25327211]
47. Tseng CW, Liu GY. Expanding roles of neutrophils in aging hosts. *Current opinion in immunology* 2014; 29:43–8. [PubMed: 24776646]
48. Aden K, Rehman A, Falk-Paulsen M, et al. Epithelial IL-23R Signaling Licenses Protective IL-22 Responses in Intestinal Inflammation. *Cell reports* 2016; 16:2208–18. [PubMed: 27524624]

49. Flannigan KL, Ngo VL, Geem D, et al. IL-17A-mediated neutrophil recruitment limits expansion of segmented filamentous bacteria. *Mucosal immunology* 2017; 10:673–84. [PubMed: 27624780]

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Highlights

- Middle-aged mice exhibit increased susceptibility to *Clostridium difficile* infection.
- Susceptibility was associated with an impaired innate immune response.
- Levels of IL-22 were significantly lower in middle-age mice.
- Administration of IL-22 during infection reduced age-related morbidity and mortality.

FIG. 1. Susceptibility of middle-aged mice to *C. difficile* infection.

(A, B) Young (1.5–2 months; solid circles, dashed line) and middle-aged (12–14 months; gray circles, solid line) C57BL/6 mice were orally infected with 10^3 *C. difficile* spores (n=16 mice/group). (A) Change in initial body weight over time after infection. Data are presented as mean $\pm$ SE of three separate experiments; * p < 0.05 vs. young mice. (B) Percentage of infected mice exhibiting 2 clinical symptoms (20% body weight loss, blood present in the stool, watery diarrhea). * p < 0.05 vs. young mice. (C, D) Stratification of weight loss and symptom-free survival by age and sex. Aged females (gray triangles, solid line; n=6), aged males (gray squares, dash-single dot line; n=10), young females (solid triangles, dashed line; n=10), young males (solid squares, dash-double dot line; n=6). * p < 0.05 vs. sex-matched young mice.

FIG.2. Effect of age on host defenses against *C. difficile*.

Young (Y, solid bars) and middleaged (M, gray bars) mice were infected with *C. difficile*.

(A) *C. difficile* numbers (left) and toxin titers (right) in colon contents of young (n=6) and middle-aged mice (n=9) 7 days after infection. Data are mean $\pm$ SE from 3 independent experiments. (B) Bacterial numbers in MLN, spleen, liver, and kidneys determined by CFU assay on BHI agar. Data are mean $\pm$ SE. The horizontal dashed lines depict the detection limit of the assays. * $p < 0.05$ vs. young mice.

FIG.3. Comparison of microbial community composition in mice following CDI.

(A) Alpha diversity, as measured by observed OTUs, was assessed using cecal contents from young (Y, black circles) and middle-aged (M, gray circles) mice following antibiotic treatment (Abx) and 9 days after *C. difficile* infection (CDI). Untreated, uninfected mice served as controls. Each point represents an individual animal; *, p -value < 0.05 relative to indicated control; NS, not significant compared to young controls. (B) Family-level relative abundance changes were independent of age. (C) Mean OTU counts of OTU_11 in mice 9 days after *C. difficile* infection. Significance was determined by the Mann-Whitney U test. *, p -value < 0.05. (D) Pairwise relationships between samples were determined using abundance-adjusted Jaccard similarity index and plotted with non-metric multi-dimensional scaling (NMDS). Each point represents an individual animal.

FIG.4. Effect of age on host responses to *C. difficile*.

Young and middle-aged mice were infected with *C. difficile* or left uninfected. Colon tissues were collected 9 days after infection. (A,B) mRNA expression was analyzed by qRT-PCR (left panel) for the IL-6 and IL-23p19. Expression levels are shown relative to age-matched untreated controls. Corresponding protein levels were determined in colon extracts by ELISA (right panel). Data are means $\pm$ SE, n = 5 mice/group. *, $p < 0.05$ vs. young mice; * $p < 0.05$ vs. age-matched uninfected mice; NS, not significant. (C) Neutrophil numbers were determined on H&E stained colon sections. Data are means $\pm$ SE, n=3–9 mice/group. * $p < 0.05$ vs. young mice; * $p < 0.05$ vs. age-matched uninfected mice. (D) CXCR2, myeloperoxidase (MPO) and CXCL2 mRNA expression were determined by qRT-PCR as described above. Data are mean $\pm$ SE, n = 5 mice/group. * $p < 0.05$ vs. young mice; * $p < 0.05$ vs. age-matched uninfected mice; NS, not significant.

FIG.5. Importance of IL-22 in limiting *C. difficile*-induced damage in middle-aged mice. (A) Young and middle-aged mice were infected with *C. difficile*, and IL-22 mRNA expression (left panel) and protein (right panel) were determined in the colon 9 days after infection. Expression levels are shown relative to age-matched untreated controls. Data are mean $\pm$ SE (n = 5 mice/group). * p < 0.05 vs. young infected mice; * p < 0.05 vs. age-matched uninfected mice; NS, not significant. (B-D) Middle-aged mice treated with recombinant IL-22 (rIL-22; n=8) or vehicle control (PBS; n=6) were infected with *C. difficile*. (B) Effect of rIL-22 on disease occurrence, as defined by the presence of 2 or more symptoms. * p < 0.05 vs. PBS-treated middle-aged controls. (C, D) Colons were collected at day 3 and examined histologically. Representative H&E-stained sections are shown in panel C (scale bars, 100 μ m). Histological scores of damage were determined (D; each data point represents one animal, horizontal lines represent mean values for each group; * p < 0.05 vs. PBS-treated middle-aged mice). (E) Expression levels are shown relative to uninfected controls. Data are means $\pm$ SEM; p < 0.05 vs. PBS-treated middle-aged controls.

Table 1.

Primer sequences

Gene	Sequence (5'→3')	Length (bp)
<i>Il22</i>	Forward: GCC AGC CTT GCA GAT AAC AAC A Reverse: GCT GAG CTG ATT GCT GAG TTT GGT	159
<i>Il23p19</i>	Forward: TGG CAT CGA GAA ACT GTG AGA Reverse: TCA GTT CGT ATT GGT AGT CCT GTT A	231
<i>Il6</i>	Forward: AGA CAA AGC CAG ACT CCT TCA GAG Reverse: GCC ACT CCT TCT GTG ACT CCA GC	146
<i>Cxcl2</i>	Forward: TTT CCA GGT CAG TTA GCC TTG CCT Reverse: AAA GTT TGC CTT GAC CCT GAA GC	86
<i>Cxcr2</i>	Forward: TCT GCT CAC AAA CAG CGT CGT AGA Reverse: AGC ATC TGG CAG AAT AGA GGG CAT3	153
<i>Mpo</i>	Forward: CAG CGA GGA CCC CCT AGC CA Reverse: GGC ATC TCG CTG GAG CGC AT	198
<i>Saa1</i>	Forward: GCT GAC CAG GAA GCC AAC AG Reverse: CAG GCA GTC CAG GAG GTC TG	73
<i>Reg3γ</i>	Forward: ATG GCT CCT ATT GCT ATG CC Reverse: GAT GTC CTG AGG GCC TCT T	87
<i>Muc2</i>	Forward: CCA TTG AGT TTG GGA ACA TGC Reverse: TTC GGC TCG GTG TTC AGA G	104
<i>Muc3</i>	Forward: GGA ACT GGT GGA GAG CGT AG Reverse: CGG TCC TGT AGC TTC TCA CTG	102
<i>Gapdh</i>	Forward: GTG CAG TGC CAG CCT CGT CC Reverse: GCC ACT GCA AAT GGC AGC CC	102