Gut microbiome communication with bone marrow regulates susceptibility to amebiasis

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The microbiome provides resistance to infection. However, mechanisms for this are poorly understood. Here we demonstrate in a murine model that colonization with the intestinal bacterium *Clostridium scindens* provided protection from *Entamoeba histolytica* colitis via innate immunity. Introduction of *C. scindens* into the gut microbiota epigenetically altered and expanded bone marrow granulocyte-monocyte-progenitors (GMPs) and resulted in increased intestinal neutrophils with subsequent challenge with *E. histolytica*. Introduction of *C. scindens* alone was sufficient to expand GMPs in gnotobiotic mice. Adoptive transfer of bone-marrow from *C. scindens* colonized-mice into naïve-mice protected against amebic colitis and increased intestinal neutrophils. Children without *E. histolytica* diarrhea also had a higher abundance of Lachnoclostridia. Because of the known ability of the Lachnoclostridia *C. scindens* to metabolize the bile salt cholate, we measured deoxycholate and discovered that it was increased in the sera of *C. scindens* colonized specific pathogen free and gnotobiotic mice, as well as in children protected from amebiasis. Administration of deoxycholate alone (in the absence of *C. scindens*) increased GMPs and provided protection from amebiasis. We have discovered a mechanism by which *C. scindens* and the microbially-metabolized bile salt deoxycholic acid alter hematopoietic precursors and provide innate protection from later infection with *Entamoeba histolytica*.
Gut microbiome communication with bone-marrow regulates susceptibility to amebiasis.

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Abstract:

The microbiome provides resistance to infection. However, mechanisms are poorly understood. We demonstrate that colonization with the intestinal bacterium *Clostridium scindens* protects from *Entamoeba histolytica* colitis via innate immunity. Introduction of *C. scindens* into the gut microbiota epigenetically altered and expanded bone-marrow granulocyte-monocyte-progenitors (GMPs) and resulted in increased intestinal neutrophils with subsequent challenge with *E. histolytica*. Introduction of *C. scindens* alone was sufficient to expand GMPs in gnotobiotic mice. Adoptive transfer of bone-marrow from *C. scindens* colonized-mice into naïve-mice protected against amebic colitis and increased intestinal neutrophils. Children without *E. histolytica* diarrhea also had a higher abundance of Lachnoclostridia. *Lachnoclostridia C. scindens* can metabolize the bile salt cholate, so we measured deoxycholate and discovered that it was increased in the sera of *C. scindens* colonized SPF and gnotobiotic mice, as well as in children protected from amebiasis. Administration of deoxycholate alone increased GMPs and provided protection from amebiasis. We elucidated a mechanism where *C. scindens* and the microbially-metabolized-bile salt deoxycholic acid alter hematopoietic precursors and provide innate protection from later infection with *Entamoeba histolytica*.
Introduction

Commensal intestinal bacteria may protect from infection (1,2) by modulating bone-marrow production of innate immune effector cells including neutrophils and inflammatory macrophages (3–5). The host metabolome is influenced by the composition of the commensal gut microbiome, and is implicated in communicating and directing the development of innate immunity, to some extent, via bile acids (6). Primary bile acids produced by the host and secondary bile acids metabolized by the intestinal microbiota (e.g. deoxycholic and lithocholic acid), can act as signaling molecules, much like host damage-associated molecular pattern molecules (DAMPs)(6). Bile acids within the intestine may protect from intestinal pathogen infection (7). Bile acid receptors are expressed in many cells implicated in innate immunity, are present in the myeloid lineage, and may impact expansion of these cells (8). Bone-marrow also has the ability to recognize bile acids (9,10). Epigenetic effects may result from signaling via bile acids, including inducing methyltransferase activity (11). This may explain in part how infection with one microorganism alters the inflammatory response to other pathogens, providing innate protection from infection with unrelated pathogens (12–15).

Epigenetic changes, such as H3K27 and H3K4 methylation associated with promotor regions of innate inflammatory genes (16–18), have been implicated as a mechanism for this process. As such, commensal microbial metabolite alteration of H3K27 demethylase expression in innate immune populations might contribute to protection from infection (17,19). Host DAMPs that can be systemically induced by the microbiota have also been shown to be important in upregulating demethylase expression in myeloid cell lines and mouse bone-marrow (12,20). Collectively, these data suggest a role of serum soluble mediators, including secondary bile acids, induced by the microbiota in communicating to the bone-marrow to influence immunity to infection. We
sought here to better understand the mechanism by which protective immunity induced by a metabolic product of the microbiota might occur during infection with a human intestinal pathogen.

Methods
Supplemental materials include full methods. Sequencing data is contained in the GEO repository under accession number GSE121503, the SRA under accession number PRJNA503904 and under SRA and linked via the dbGaP accession number phs001478.v1.p1. Data is available in the manuscript and supplement. Design of the human cohort studies have been described (21,22) and all studies were approved by the Research and Ethical Review Committees of the icddr,b and the Institutional Review Boards of the University of Virginia. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Virginia. All experiments were performed according to provisions of the Animal Welfare Act of 1996 (§ 89.544).

Results and Discussion
Previous work suggested that murine commensals influence the inflammatory capacity of marrow derived cells (12,13). We hypothesized that components of the human gut microbiota might alter bone-marrow hematopoiesis to confer protection against unrelated pathogens such as Entamoeba histolytica (23,24). To explore this possibility, we first tested for human commensals associated with protection from amebiasis. Principal coordinates analysis of beta-diversity indicated that the microbiota of children with E. histolytica diarrhea differed significantly (Figure 1 A) with a decrease in the relative abundance of the genus Lachnoclostridium (Figure 1 B). Some Lachnoclostridium are known to alter the metabolome, including the bile acid pool
of the intestine (7,25,26). We hypothesized that these bacteria provide protection from Entamoeba. To test this hypothesis we introduced a member of the Lachnoclostridium genus, the human commensal bacteria Clostridium scindens (26) into the gut microbiome of susceptible CBA/J mice (27) and challenged them with the parasite E. histolytica.

C. scindens was significantly increased in the microbiota after gavage, and gut community structure was also altered (Figure S5, A, B). Introduction of C. scindens to the gut microbiome provided protection from E. histolytica (Figure 1 D,J, Figure S4 B, S5 E) and this protection was associated with increased intestinal neutrophil infiltration (Figure 1 E, K, Figure S3 B). This increase in gut neutrophils only occurred with Entamoeba infection (Figure 1 C, E). There was no significant difference in intestinal CD4+ and CD8+T-cells, eosinophils or inflammatory monocytes (Figure 1 F-I) in C. scindens colonized mice.

Myeloid cell expansion may be influenced by cytokine production by CD8+T-cells (28) or intestinal T regulatory cells (29). Contribution of the acquired immune system to C. scindens-mediated protection was tested by using RAG-1−/− mice, which lack B and T-cells. RAG-1−/− mice were also protected from E. histolytica when colonized with C. scindens (Figure 1 J, K) indicating that protection did not require the acquired immune system.

The increase in gut neutrophils in response to Entamoeba infection in C. scindens colonized mice suggested that C. scindens may have altered innate bone-marrow populations that give rise to neutrophils. Therefore we examined hematopoietic progenitors in C. scindens colonized specific-pathogen-free mice (SPF) (Figure 2 A, B), SPF RAG-1−/− mice (Figure 2 D) and C. scindens gnotobiotic mice and germ-free controls (Figure 2 C). Intestinal colonization with C. scindens increased bone-marrow granulocyte progenitor cells (Figure 2). Expansion of GMPs mediated by C. scindens occurred in the absence of T-cells (Figure 2 D) and colonization with
C. scindens alone was sufficient to increase marrow GMPs as demonstrated by our study in gnotobiotic animals (Figure 2 C). This suggested that innate immune cells underlie the observed C. scindens mediated changes in hematopoiesis and protection from Entamoeba. The increase in intestinal neutrophils in C. scindens colonized mice only occurred following Entamoeba challenge. This suggested there may be homeostatic changes in pathways in GMPs important in neutrophil production (5).

To explore this possibility, we examined transcriptional and epigenetic changes in marrow GMPs from C. scindens colonized mice. Gene enrichment analysis of RNA sequencing data suggested that genes associated with covalent modification of the histone H3 tail, such as the demethylase JMJD3, were enriched in mice exposed to C. scindens (Figure S3 F). This analysis also indicated enrichment of genes associated with CCAAT/enhancer-binding proteins, known to be important for GMP and neutrophil differentiation and expansion (30,31) (Figure S3 F). QPCR of sorted marrow GMPs confirmed that significant changes in expression occurred in JMJD3 (Figure S3, G, J, K) and two CCAAT/enhancer-binding-protein genes important in granulopoiesis (30,31), CEBPA (Figure S3, H), and CEBPB (Figure S3, I). Therefore we examined H3K4me3 and H3K27me3 occupancy in the promoter regions of CEBPA and CEBPB, in sorted GMPs. The repressive mark H3K27me3 was decreased in the promoter of CEBPA in C. scindens colonized mice (Figure S3 L) while the activating mark H3K4me3 (Figure S3 M) was increased in the promoter of CEBPB in C. scindens colonized mice. Therefore, bone-marrow epigenetic alteration occurred with gut colonization by C. scindens. This suggested bone-marrow changes might underlie gut-immunity to Entamoeba in colonized mice. To explore this possibility we utilized adoptive marrow transplants.
Adoptive transfer of bone-marrow from \textit{C. scindens} colonized mice into mice not previously exposed to \textit{C. scindens} was sufficient to provide protection from \textit{E. histolytica} (Figure 3 A) as well as recapitulate the increase in marrow GMPs (Figure 3 B) and intestinal neutrophils (Figure 3 C). In contrast, previous epithelial exposure to \textit{C. scindens} was not sufficient to provide protection from ameba in irradiated mice (Figure 3A). We also noted an overall increase in GMPs in the mice post bone-marrow transplant: however, this increase was controlled across all groups and was likely a response to irradiation (32). We concluded that alterations in radio-sensitive marrow hematopoietic cells caused by gut exposure to \textit{C. scindens} were sufficient to confer protection to a later \textit{E. histolytica} challenge.

We next explored how intestinal colonization with \textit{C. scindens} could be altering GMPs in the bone-marrow. \textit{C. scindens} is known to be capable of 7α-dehydroxylation of bile acids in the intestine (26). Gavage and colonization with another human mucosal anaerobic bacterium lacking 7α-dehydroxylation activity did not induce protection from \textit{Entamoeba} (Figure S5, C-E). Colonization of mice with \textit{C. scindens} was sufficient to increase serum levels of the secondary bile acid deoxycholic acid (DCA, a product of 7α-dehydroxylation of cholic acid) in SPF and in gnotobiotic mice (Figure 4 A, Figure S2 A, B). Absolute levels of DCA were lower in gnotobiotic mice in both groups than in SPF however. This is perhaps due to the lack of other members of the microbiota producing products upstream of 7α-dehydroxylation (33). DCA was also increased in children (from two independent cohorts) protected from \textit{E. histolytica} (Figure 4 B, Figure S1 A, B). We concluded that DCA in plasma was positively correlated with protection from \textit{Entamoeba} in the mouse model of amebic colitis and in children. Future studies may examine these effects in adult patients.
To test if transient elevation of serum DCA was sufficient to mediate protection from *Entamoeba* we administered the bile salt intravenously. Administration of DCA prior to *Entamoeba* infection increased serum levels of deoxycholate (Figure 4 **C, D**) and provided protection from infection (Figure 4 **E**). Treatment of mice with DCA was not associated with elevated markers of liver damage or intestinal inflammation prior to *Entamoeba* infection (Figure S6). Protection from *Entamoeba* was associated with increased marrow GMPs and gut neutrophils (Figure 4 **F, G**). Experimental elevation of serum DCA increased expression of the epigenetic mediator JMJD3 in sorted marrow GMPs (Figure S3 **K**). We concluded that DCA was sufficient to recapitulate the changes in GMPs and protection from *Entamoeba* afforded by *C. scindens*. This work provides observations by which *C. scindens* and the microbially-metabolized bile salt DCA are sufficient to alter hematopoietic precursors and provide innate protection from later infection. These studies however do not rule out the contribution of other bile acids and metabolites to gut to bone-marrow communication.

Deoxycholate-mediated protection from *E. histolytica* was associated with increased marrow GMPs and intestinal neutrophils as seen with *C. scindens*. We next explored pathways by which deoxycholate or *C. scindens* might increase GMPs. Due to the epigenetic changes observed (Figure S3 **L, M**), persistent nature of immunity to *E. histolytica* following bone-marrow transplant in the absence of colonization with the commensal (Figure 3), and upregulation of JMJD3 in sorted marrow from *C. scindens* colonized or DCA treated mice (Figure S3 **G, J, K**), we examined the role of JMJD3 activity during *C. scindens* colonization on protection from *Entamoeba* infection. Treatment with an inhibitor of JMJD3 during *C. scindens* colonization abrogated bone-marrow GMP expansion (Figure S4 **A**) as well as induction of intestinal neutrophils and protection from *E. histolytica* (Figure S4 **B, C**). This suggests H3K27
demethylase activity may contribute to gut to marrow communication by *C. scindens*. JMJD3 is an H3K27me3 demethylase (17); however, we also observed changes in H3K4me3 in the promoter region of CEBPB. JMJD3 has recently been shown to impact H3K4me3 levels in human acute myeloid leukemia (AML) cells (34). This may not fully explain the epigenetic changes in our model, and other epigenetic mediators, including other non-methyl modifications, might influence gut microbiota mediated communication with the bone-marrow.

The results presented here suggest a model whereby gut colonization with *C. scindens* increases serum deoxycholate that then acts on the marrow to increase transcription of genes that support granulocyte monocyte progenitor (GMP) expansion, such as CCAAT/enhancer-binding-proteins CEBPA and CEBPB. Then, when a different challenge occurs at a mucosal site (in this case infection with *E. histolytica*), a more robust neutrophil response results.

Future studies will examine the precise mechanisms by which *C. scindens* colonization alters bone-marrow hematopoiesis, which are not fully elucidated by these studies. However this work yields understanding of how changes in the gut microbiome can result in antigen nonspecific protection from *Entamoeba histolytica* infection. This heightened inflammatory response may have implications for other infectious diseases, and potentially other mucosal sites in the body such as the lung. Therefore, the impact of the work extends beyond infectious disease to fundamental mechanisms of gut-to-bone-marrow communication by commensal bacteria. These studies may help in development of microbiome-targeted treatments that modulate the severity of immune and inflammatory diseases by altering bone-marrow production of inflammatory cells.

**Author’s contributions.** SLB, JLL, JU, NO, KW, MS, NS, NG, BAT and BM performed experiments. SLB, JLL, DTA, ST, DO, NG, JZM, ZP, BM and BAT analyzed the data. WAP,
SLB, JS, RH, JZM, supervised the experiments and data analysis. SLB and WAP developed the theoretical framework. All authors discussed the results and contributed to the preparation of the manuscript.

Acknowledgments

We thank Tuhinur Arju, and Mamun Kabir at icddr, b, at UVA, Jeremy Gatesman, Homer Ransdell, Alice Kenney and Sanford Feldman at the Center for Comparative Medicine, Michael Solga, Claude Chew, and Joanne Lannigan at the Flow Cytometry Core facility, AhnThu Nguyen at the Biology Department Genomics Core, and Katia Sol-Church and Alyson Prorock at the Genome Analysis and Technology Core, Todd Fox at the Metabolomics Core, and Epigentek, NY, for technical support. We thank Emery H. Bresnick at UW-Madison for helpful scientific discussions.

Funding. The work was supported by National Institutes of Health National Institute of Allergy and Infectious Diseases Grants R01 AI-26649 and R01 AI043596 (WAP), T 32 5T32AI007046. by the Bill and Melinda Gates Foundation, by Robert and Elizabeth Henske and 1R21AI130700 (SLB).

Competing interests. Authors declare no competing financial interests
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Figure 1. *Lachnoclostridium* are associated with protection from *Entamoeba histolytica* in children, and introduction of the *Lachnoclostridium* *Clostridium scindens* to the gut microbiota provides innate protection from *Entamoeba histolytica* in a murine model. (A) Principal Coordinates Analysis (PCoA) of Bray-Curtis dissimilarities (beta-diversity) of fecal microbiota from surveillance reference stool or *E. histolytica* infected children was performed. The groups are significantly different by PERMANOVA, p<0.05. (B) Relative abundance of the genus *Lachnoclostridium* from samples described in A. The groups are significantly different by Wilcoxon rank sum test with continuity correction, p<0.05. (A, B), N= 20 children per condition. CBA/J mice (C-I) or C57BL/6 RAG1 -/- mice (J,K) were colonized with bile acid 7α-dehydroxylating bacteria *C. scindens* (ATCC® 35704) over three weeks prior to intracecal infection with *E. histolytica*. (C) Gut neutrophil infiltration was determined prior to ameba infection via flow cytometry. (D, J) Percent of mice infected with *Entamoeba* at day six following infection was determined via cecal culture in trophozoite culture media. (E-I, K) Gut immune cell infiltration was determined via flow cytometry. * p<0.05, (C, E-I, K), Student's t-test, (D, J), Mann–Whitney U test, bars indicate mean, N = 4-9 mice per group.
Figure 2. Intestinal colonization with *C. scindens* expands bone-marrow granulocyte monocyte progenitors. (A, B) CBA/J, SPF, mice, (C) Gnotobiotic C57BL/6 or (D) SPF C57BL/6 RAG1 -/- mice were colonized with bile acid 7α-dehydroxylating bacteria *C. scindens* (ATCC® 35704). (A, C, D) Flow cytometry and (B) colony forming assays were utilized to determine composition of marrow hematopoietic precursors in *C. scindens* colonized CBA/J or RAG1-/- mice. Common Myeloid Progenitors (CMP) are Lin-c-Kit+Sca-1-CD34+FcgRII-IIIint; Granulocyte-Monocyte-Progenitors (GMP) are Lin-c-Kit+Sca-1-CD34+FcgRII-IIIhi; Megakaryocyte–Erythroid Progenitors (MEP) are Lin-c-Kit+Sca-1-CD34-FcgRII-III-. Colony formation in B was assayed of burst-forming unit–erythroid (BFU-E), colony-forming unit–granulocyte/monocyte (CFU-GM), and CFU granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM)* p<0.05, Student's t-test, bars and whiskers are mean and Tukey method. N =6-8 mice per group.
Figure 3. Bone-marrow from *C. scindens* colonized donors is sufficient to provide protection from *Entamoeba* in *C. scindens* naïve mice. CBA/J mice colonized with *C. scindens* (+) or not (-) were lethally irradiated and given whole marrow from *C. scindens* (+) or *C. scindens* (-) donors then allowed to recover for 7 weeks prior to *Entamoeba* challenge. (A) Protection from amebic colitis, (B) change in marrow GMPs, and (C) gut neutrophil infiltration were determined at 8 weeks post BMT. *= p<0.05, (A) Mann–Whitney U test, (B, C) One Way ANOVA with Tukey post-test, bars and whiskers are mean and Tukey method. N = 4-8 mice per group.
Figure 4. *C. scindens* colonization increases serum deoxycholic acid (DCA), and administration of DCA expanded marrow GMPs, intestinal neutrophils and protected from amebic colitis. (A) CBA/J mice were colonized with *C. scindens* over three weeks via gavage and serum DCA was measured at 10 weeks of age in control BHI media gavaged mice and *C. scindens* gavaged mice. (B) Serum DCA was measured via ELISA in 2 year old children in Bangladesh free of (-) or infected with (+) *E. histolytica* within 6 months of the blood draw. Mice were administered DCA or PBS intravenously three times a week for two weeks and then challenged with *E. histolytica*. Serum DCA was measured and the end of week 1 (Day -7) (C), and at the end of the experiment (Day 6) (D). (E) *E. histolytica* infection, (F) change in marrow GMPs and (G) intestinal neutrophils were measured at the end of the experiment. *= p<0.05, (A-D, F, G) Student’s t-test, (E) Mann–Whitney U test. N =6-8 mice per group. (B) N= 40 children per condition.