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CCR2-dependent CX3CR1+ colonic macrophages promote *Enterococcus faecalis* dissemination

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ABSTRACT Enterococci are common commensal bacteria that colonize the gastrointestinal tracts of most mammals, including humans. Importantly, these bacteria are one of the leading causes of nosocomial infections. This study examined the role of colonic macrophages in facilitating *Enterococcus faecalis* infections in mice. We determined that depletion of colonic phagocytes resulted in the reduction of *E. faecalis* dissemination to the gut-draining mesenteric lymph nodes. Furthermore, we established that trafficking of monocyte-derived CX3CR1-expressing macrophages contributed to *E. faecalis* dissemination in a manner that was not reliant on CCR7, the conventional receptor involved in lymphatic migration. Finally, we showed that *E. faecalis* mutants with impaired intracellular survival exhibited reduced dissemination, suggesting that *E. faecalis* can exploit host immune cell migration to disseminate systemically and cause disease. Our findings indicate that modulation of macrophage trafficking in the context of antibiotic therapy could serve as a novel approach for preventing or treating opportunistic infections by disseminating enteric pathobionts like *E. faecalis*.

KEYWORDS *Enterococcus faecalis*, colon, dissemination, macrophages, CX3CR1, CCR2, gut-associated lymphoid tissue, commensal, migration, pathobiont

Enterococci are Gram-positive commensal bacteria found in the gastrointestinal tract of most mammals. Although enterococci are typically non-pathogenic, they are intrinsically resistant to cephalosporin antibiotics, and treatment with cephalosporins can lead to opportunistic infections in humans and mice (1, 2). Currently, enterococci are the third leading cause of infectious endocarditis, with *Enterococcus faecalis* contributing to >90% of reported enterococcus-related cases (3, 4). Furthermore, the rising prevalence of vancomycin-resistant enterococci, predominantly *Enterococcus faecium*, has made enterococci a leading cause of hospital-acquired infections in the United States (5). Nevertheless, most individuals colonized with enterococci, including *E. faecalis* or *E. faecium*, do not succumb to infections (6, 7). This highlights the generally commensal nature of enterococci but suggests a gap in our understanding of the preconditions for opportunistic infections by these pathobionts. To date, the mechanisms by which commensal enterococci subvert host immunity to become pathogenic remain poorly understood.

The co-evolution between the host and commensals has resulted in immune mechanisms designed to tolerate, and even benefit from, gut-colonizing bacteria. Antigen-presenting cells (APCs) like macrophages and dendritic cells (DCs) of the gut-associated lymphoid tissue (GALT) selectively sample luminal microorganisms to educate the adaptive immune system and maintain homeostasis. For instance, the CX3CR1-dependent extension of transepithelial dendrites into the intestinal lumen or the passage of bacteria via goblet cells and microfold cells to underlying APCs represents coordinated processes of bacterial translocation across the epithelial barrier (8–12). Upon antigen uptake and processing, APCs migrate to the gut-draining mesenteric lymph

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nodes (MLN) where immunological tolerance to commensal antigen is generated (13–15).

A growing body of evidence suggests that APC migration can contribute to the transport of live bacteria from the gut to the MLNs, a process that may serve homeostatic functions. For instance, delivery of live *Enterobacter cloacae* to the MLNs by CD11c-expressing leukocytes promotes the production of IgA that protects mice from excessive microbial dissemination (16). Moreover, the presence of commensal bacteria in the MLNs at a steady state has been shown to induce low levels of circulating IgG that provides cross-reactive protection against challenges with lethal doses of *E. coli* or *Salmonella* (17). Although the mechanism by which these commensals arrived in the MLNs was not addressed in that study, the possible contribution from intestinal APCs should not be overlooked.

Many bacterial species are inherently well suited for intracellular survival within phagocytes (18–21) which likely contributes to their inadvertent transport to the MLN via migratory APCs. This phenomenon is well described in *Salmonella typhimurium* infections (22–25), whereby *Salmonella* subverts APC killing mechanisms while it is trafficked to the MLNs. This type of APC-mediated infection has also been observed with other pathogenic microbes like *Listeria monocytogenes* in the gut and *Staphylococcus aureus* in the skin (18, 26), suggesting that APC migration is a common mechanism by which pathogens gain access to peripheral tissues and cause disease. While several intracellular survival-related pathways have been described in enterococcal species (19, 27–30), the role that intracellular APC survival plays in enterococcal dissemination from the gut remains poorly understood.

In the present study, we leveraged experimental mouse models to investigate the hypothesis that intestinal APCs contribute to enterococcal infections. We established that colonic phagocytes are required for robust *E. faecalis* dissemination to the colon-draining MLN (cMLN). We also demonstrated that *E. faecalis* dissemination relied on CCR2-dependent recruitment of CX3CR1+ macrophage precursors, but not lymphatic migration of CCR7+ conventional DCs. Importantly, this dissemination was shown to rely on intracellular survival mechanisms employed by *E. faecalis* in both mono-colonized germ-free (GF) and ceftriaxone-treated mice. Our observations show that intracellular survival within colonic macrophages contributes to *E. faecalis* escape from the gut and dissemination into systemic circulation.

RESULTS

E. faecalis disseminate from the colon to the colon-draining mesenteric lymph node

The MLNs consist of several individual lymph nodes that drain distinct regions of the GI tract (31). We previously reported that ceftriaxone-induced dysbiosis led to *E. faecalis* overgrowth and subsequent dissemination to the MLNs (32); however, we did not distinguish between cMLN and small intestine-draining MLNs (siMLNs). Others have shown that broad microbial depletion using antibiotics led to dissemination of colonic bacteria, including *E. faecalis*, to the cMLN (12). Therefore, we adapted our previously reported ceftriaxone-induced *E. faecalis* dissemination model (2, 32) (Fig. 1A) to test whether *E. faecalis* dissemination was restricted to the cMLN in ceftriaxone-treated mice. We determined that siMLN dissemination was rarely observed and that the majority of *E. faecalis* disseminates to the cMLN (Fig. 1B), suggesting that ceftriaxone-mediated escape occurs from the colon. Furthermore, we observed a significant correlation between colonic *E. faecalis* abundance and its dissemination to the cMLN which was not observed when comparing with the contents from the distal small intestine (SI) or cecum (Fig. 1C; Fig. S1A through C). This confirms previous speculations put forth by us and others that enterococcal abundance within the gut contributes to its dissemination (2, 33). To determine whether *E. faecalis* dissemination requires antibiotic intervention, we colonized GF mice with *E. faecalis* and assessed dissemination after 7 days (Fig. 1D). While, in this case, *E. faecalis* was detected in the siMLN of GF mice, we observed

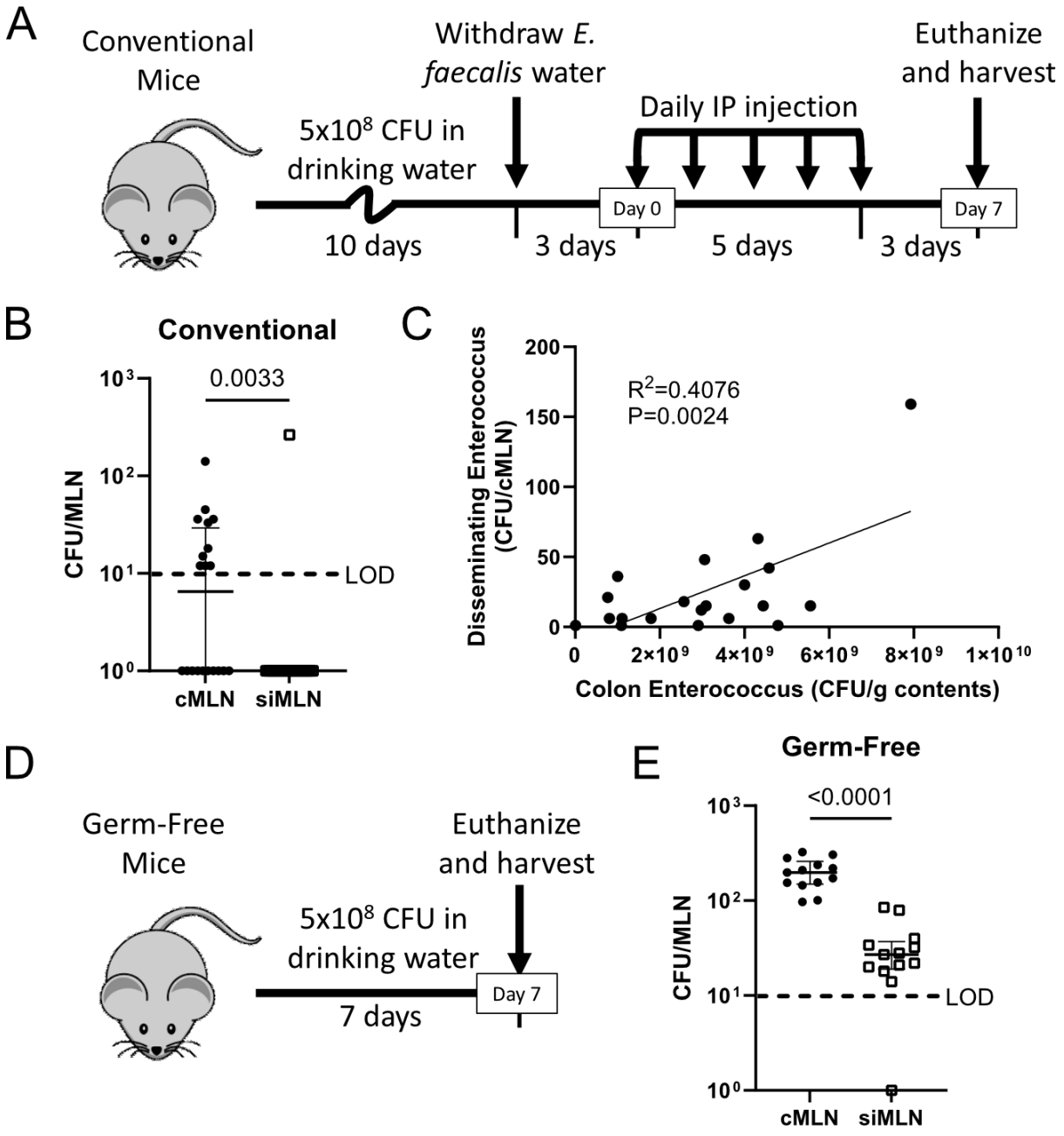

FIG 1 *E. faecalis* disseminate from the colon to the cMLN. (A) Experimental timeline for ceftriaxone-induced *E. faecalis* dissemination model. (B) The cMLN and siMLNs were collected separately, homogenized, and plated on selective agar to enumerate *E. faecalis* dissemination. (C) Colon contents were collected and plated to enumerate colonic *E. faecalis* abundance (x-axis) and plotted against cMLN dissemination (y-axis). (D) Experimental timeline for germ-free *E. faecalis* dissemination model. (E) *E. faecalis* dissemination to the cMLN and siMLN of germ-free mice was quantified as in A. (B and E) Median and interquartile range are reported, and *P* values were calculated using a Mann-Whitney test. Data are pooled from three independent experiments, using three to five mice each. (C) Data are pooled from four independent experiments using five mice per group. R^2 and *P* values were calculated using a Pearson correlation analysis. LOD, limit of detection (10 CFU/organ); $P > 0.05$ not reported.

significantly more dissemination to the cMLN (Fig. 1E). This suggests that *E. faecalis* preferentially disseminates from the colon to the cMLN, irrespective of antibiotics or the presence of competing microbiota.

Ceftriaxone treatment does not elicit gross immunological changes in the colon

We previously reported that ceftriaxone does not lead to intestinal permeability or pathology (2). Others have determined that certain antibiotics induce goblet cell-associated passages that facilitate bacterial translocation and promote colonic inflammation (12). To further clarify ceftriaxone's effects on intestinal immune responses, we compared colonic immune cell populations of ceftriaxone- and saline-treated mice. Our results indicate that ceftriaxone does not elicit gross immunological changes in colonic lymphocytes or APCs (S2A and B). Furthermore, we did not detect changes in any of the colonic lamina propria non-B cell APC subsets, including CD11c+ DCs, CD103+ DCs, CX3CR1-int macrophage/DCs, and CX3CR1-hi macrophages (S2C), subsets previously reported to migrate under various conditions (15, 16, 24, 34–36). Together, these data suggest that ceftriaxone treatment induces *E. faecalis* dissemination in the absence of changes in lymphocyte or APC populations.

Depleting colonic phagocytes abrogates *E. faecalis* dissemination

APCs have been widely studied for their involvement in preventing bacterial invasion at mucosal surfaces. However, APCs have also been reported to contribute to the dissemination of commensals and pathogens (12, 16, 22–25). To investigate the role of intestinal APCs in *E. faecalis* dissemination, we depleted colonic phagocytes by rectally administering clodronate liposomes (37, 38) in the context of our dissemination model (Fig. 2A). We note that colons from mice treated with clodronate consistently yielded higher total cell numbers (S3A), making comparisons between absolute cell numbers difficult to interpret. Nonetheless, rectal clodronate clearly reduced colonic CD45+ MHCII+ CD3− CD19− APC frequencies, but not CD45+ CD3− CD19+ B cells or CD45+ MHCII− CD3+ CD19− T cells (Fig. 2B; Fig. S3B through F). Within the non-B cell APC pool, we observed broad depletion of nearly all APC subsets (Fig. 2C; Fig. S3G), indicating that these cells display phagocytic capabilities in the colon. While colonic *E. faecalis* abundance was unaffected by clodronate treatment (Fig. 2D), we observed reduced *E. faecalis* dissemination in mice whose colonic phagocytes were depleted (Fig. 2E). Since none of the assayed MHCII− myeloid cell subsets was reduced following clodronate administration (S3H), we deduced that one or more of the non-B cell MHCII+ APC subsets are responsible for promoting *E. faecalis* dissemination during ceftriaxone treatment.

E. faecalis dissemination does not rely on CCR7+ DC migration to the MLNs

Intestinal DC migration from the lamina propria to the MLNs has been shown to contribute to the dissemination of both commensals and pathogens under various circumstances (12, 16, 22–25). This migration was consistently shown to rely on CCR7, the canonical chemokine receptor involved in lymphatic migration of DCs (36, 39). Thus, we sought to test whether CCR7-dependent DC migration was responsible for *E. faecalis* dissemination to the MLN. To address this question, we administered anti-CCR7 antibodies (αCCR7) in the context of our *E. faecalis* dissemination model (Fig. 3A). Flow cytometry of MLN APCs (Fig. 3B) revealed that CCR7 blockade selectively reduced CD11c+ and CD103+ DCs in the MLN (Fig. 3C and D), supporting the role of CCR7 in recruiting CD11c+ and CD103+ DCs from the gut to the MLN. Unexpectedly, however, treatment with αCCR7 did not prevent *E. faecalis* dissemination (Fig. 3E), suggesting that CCR7-mediated DC migration is not responsible for *E. faecalis* delivery to the MLN.

CCR2-dependent CX3CR1+ macrophages facilitate *E. faecalis* dissemination

Our observation that colonic phagocyte depletion reduced *E. faecalis* dissemination suggests that one or more macrophage or DC subsets facilitate *E. faecalis* translocation across the epithelial barrier and/or transport to the colon-draining MLN. Since abrogating CCR7-dependent trafficking to the MLN did not have impact on *E. faecalis* dissemination, we ruled out conventional DC migration as a potential mechanism. Thus,

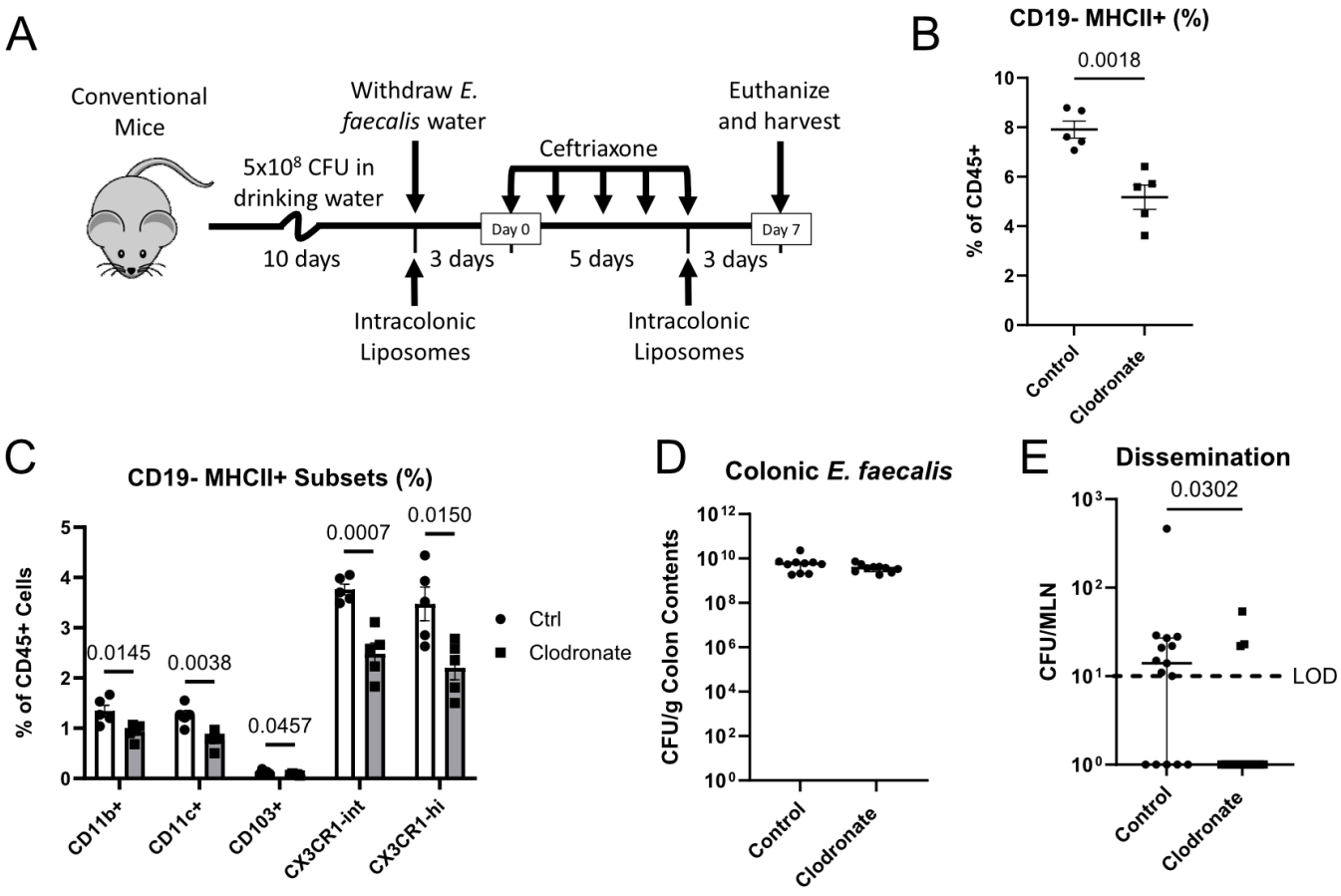

FIG 2 Clodronate-mediated depletion of colonic phagocytes reduces *E. faecalis* dissemination. (A) Experimental timeline for intracolonic liposome administration within our standard *E. faecalis* dissemination model. (B) Flow cytometric analysis of colonic non-B cell APCs (CD45+ CD3− CD19− MHCII+) or relevant APC subsets (C) as a percentage of CD45+ cells obtained from control (Ctrl) and clodronate-treated mice. (D) Colon contents from control or clodronate-treated mice were plated on selective agar to enumerate *E. faecalis* abundance. (E) MLNs from control or clodronate-treated mice were homogenized and plated on selective agar to enumerate *E. faecalis* dissemination. (B–D) Mean and standard error of the mean are reported, and statistical significance was determined using an unpaired *t*-test. Data are representative of three independent experiments using 5 mice (B and C) or 10 mice (D) per group. (E) Median and interquartile range are reported, and *P* values were calculated using a Mann-Whitney test. Data are pooled from three independent experiments, using four to five mice per group. LOD, limit of detection (10 CFU/organ); $P > 0.05$ not reported.

we hypothesized that intestinal macrophages were responsible for *E. faecalis* escape from the colon. Most intestinal macrophages are supplied by the constant recruitment of blood monocytes in a CCR2-dependent manner (40–43). To test whether monocyte-derived macrophages contributed to *E. faecalis* dissemination, we leveraged a chemical CCR2 antagonist, RS102895 (CCR2-a), which has been previously shown to disrupt monocyte recruitment and subsequently decrease colonic macrophage populations (44). Treatment with CCR2-a in the context of our dissemination model (Fig. 4A) markedly reduced non-B cell APCs in the colon (Fig. 4B and C). More specifically, we observed a significant decrease in number and frequency of CX3CR1-int and CX3CR1-hi APCs (Fig. 4D and E), consistent with previous work showing that CCR2-dependent monocytes give rise to CX3CR1-expressing macrophages (40, 43). Interestingly, we found that mice treated with CCR2-a exhibited significantly less *E. faecalis* dissemination when compared with injection controls (Fig. 4F). Together, these experiments indicate that monocyte-derived CX3CR1+ macrophages facilitate *E. faecalis* dissemination during ceftriaxone-mediated dysbiosis.

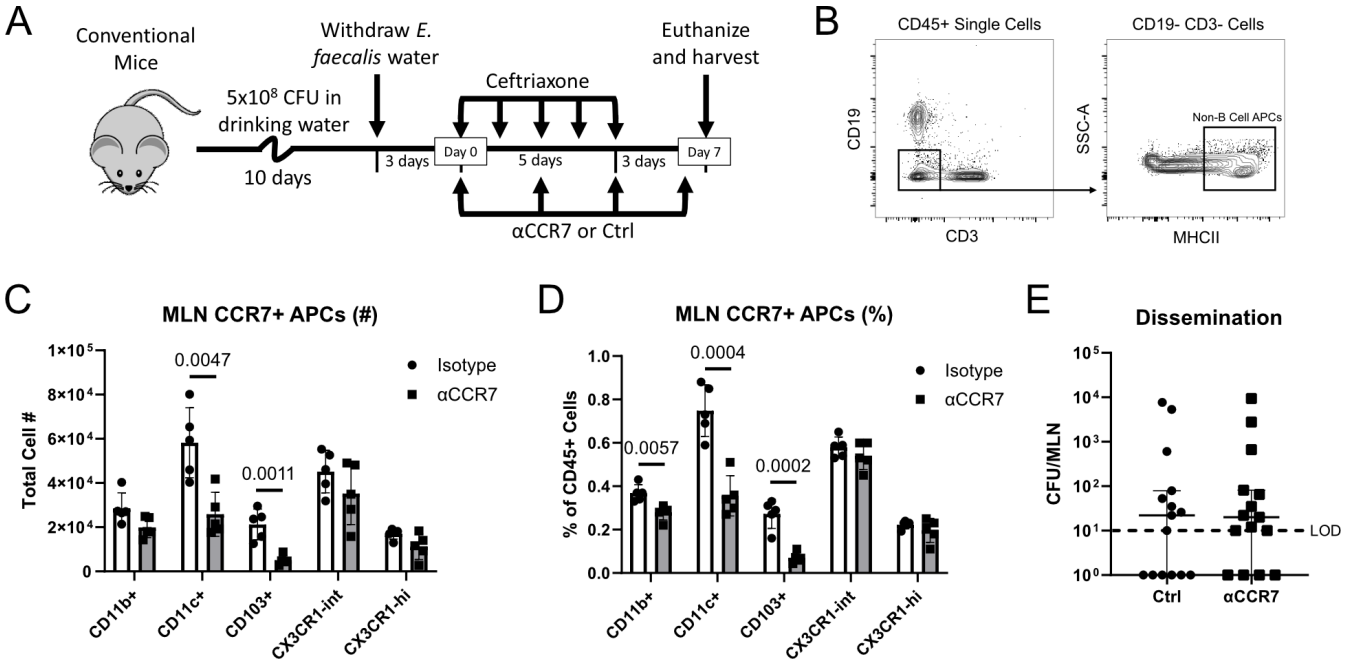

FIG 3 Inhibiting DC recruitment to the MLNs does not have impact on *E. faecalis* dissemination. (A) Experimental timeline for anti-CCR7 (αCCR7) administration within our standard *E. faecalis* dissemination model. (B) Representative gating strategy for identifying non-B cell APCs in the MLN by flow cytometry. (C and D) Flow cytometric analysis of CCR7-expressing APCs (CD45+ CD3– CD19– MHCII+ CCR7+) within the MLNs of control (isotype) or αCCR7-treated mice. Y-axis represents the total cell number (C) or the percentage of CD45+ cells (D). (E) Effects of αCCR7 treatment on *E. faecalis* dissemination in ceftriaxone-treated mice. (C and D) Data are representative of two independent experiments, using five mice per group. Mean and standard error of the mean are reported. Statistical significance was determined using an unpaired *t*-test. (E) Data are pooled from three independent experiments, using five mice per group. Median and interquartile range are reported. Statistical significance was determined using a Mann-Whitney test. LOD, limit of detection (10 CFU/organ); $P > 0.05$ not reported.

E. faecalis require oxidative stress resistance for intracellular survival but not GI tract colonization

Since *E. faecalis* is known to express the machinery needed for intracellular survival in macrophages (19, 27–30), we hypothesized that intracellular APC survival serves as a mechanism of *E. faecalis* dissemination. We confirmed these findings by co-culturing J774.A1 macrophages with *E. faecalis* [OG1RF (45, 46)] and performing a gentamicin-vancomycin protection assay to evaluate intracellular *E. faecalis* persistence over time. We showed that *E. faecalis* survives within J774 macrophages for up to 2 days, consistent with previous reports from various *in vitro* macrophage systems (19, 27–30). To ensure that these observations were not due to extracellular persistence or growth despite the presence of antibiotics, the culture media were plated to enumerate extracellular CFU. We observed minimal extracellular *E. faecalis* (<200 CFU) at 2 hours and undetectable extracellular CFU by 48 hours, confirming that *E. faecalis* persistence was due to intracellular survival (Fig. 5A).

Previous studies have identified genes that are important for *E. faecalis* survival within macrophages (19, 27–30). One such gene encodes manganese-containing superoxide dismutase (*sodA*), which catalyzes the conversion of superoxide into hydrogen peroxide and is critical for resistance to oxidative stress within the phagolysosome of macrophages (30). We leveraged an intracellular survival-deficient strain which lacks *sodA* [OG1RF Δ*sodA* (47)], to assess whether *E. faecalis* persistence within APCs contributes to its dissemination *in vivo*. First, we confirmed that deletion of *sodA* rendered *E. faecalis* more susceptible to macrophage killing using the *in vitro* system described above. We observed a modest, albeit significant reduction in Δ*sodA* mutant survival at both 24 and 48 hours post-infection when compared with the WT isogenic strain, OG1RF (Fig. 5B). Next, we assessed whether Δ*sodA* could persist long term within the GI tract of

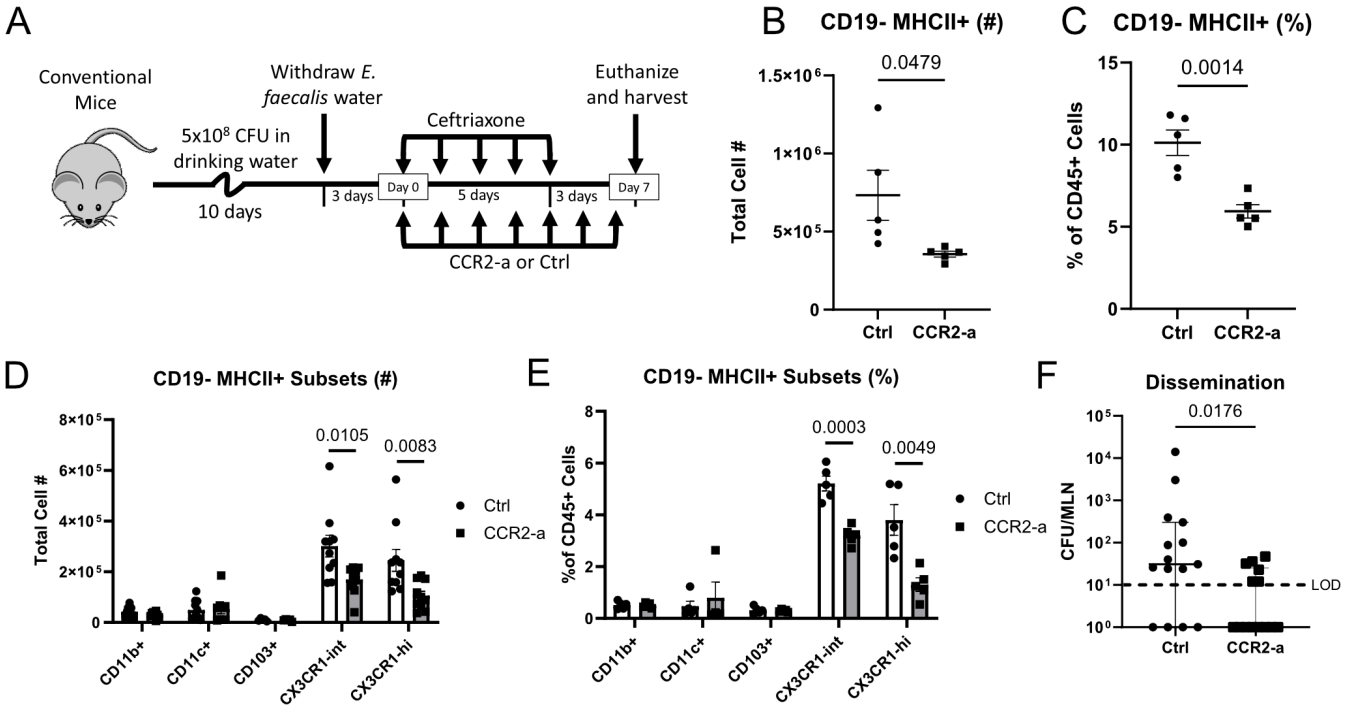

FIG 4 CCR2 antagonism reduces *E. faecalis* dissemination. (A) Experimental timeline for CCR2 antagonism (CCR2-a) within our standard *E. faecalis* dissemination model. (B and C) Flow cytometric analysis of colonic APCs (CD45+ CD3− CD19− MHCII+) and relevant APC subsets (D and E) obtained from Ctrl or CCR2-a-treated mice. Y-axis represents the total cell number (B and D) or the percentage of CD45+ cells (C and E). (F) Effects of CCR2-a on *E. faecalis* dissemination in ceftriaxone-treated mice. (B–E) Data are representative of three independent experiments, using five mice per group. (D) Data are pooled from two experiments to account for variability in cell yields. Mean and standard error of the mean are reported. Statistical significance was determined using an unpaired *t*-test. (F) Data are pooled from three independent experiments, using five mice per group. Median and interquartile range are reported. Statistical significance was determined using a Mann-Whitney test. LOD, limit of detection (10 CFU/organ); $P > 0.05$ not reported.

mice. To do this, we colonized mice as in Fig. 1A with either WT or Δ*sodA* and tracked colonization persistence in the absence of ceftriaxone treatment by enumerating fecal *E. faecalis* abundance weekly. We found that both WT and Δ*sodA* strains stably colonized mice at ~10^5 CFU/g feces (Fig. 5C), suggesting that the Δ*sodA* mutant does not exhibit colonization defects.

SodA-mediated intracellular survival is required for *E. faecalis* dissemination to the MLN

To test our hypothesis that intracellular APC survival supports *E. faecalis* dissemination, we colonized mice with WT or Δ*sodA* and treated them with ceftriaxone as in Fig. 1A to induce *E. faecalis* dissemination. Upon ceftriaxone treatment, Δ*sodA* exhibited reduced dissemination when compared with WT (Fig. 5C), suggesting that Δ*sodA* does not disseminate as efficiently. Although our previous work showed that this Δ*sodA* strain does not exhibit ceftriaxone resistance defects (47), we observed an unexpected decrease in Δ*sodA* fecal abundance during ceftriaxone treatment that was not observed in the WT strain (Fig. 5E). This initial dip was followed by a rebounding increase above colonization baseline at the time of euthanasia but was nonetheless confounding, as previous studies suggest that *E. faecalis* dissemination is correlated to its intestinal abundance (2, 33). To address this, we turned to GF mice which have been shown to permit bacterial dissemination to the MLNs during early stages of colonization (48). We colonized GF mice with WT or Δ*sodA* strains as in Fig. 1D and assessed intestinal colonization and dissemination to the MLNs after 7 days. Although similar fecal and intestinal abundance was observed between strains throughout the experiment (Fig. 5F), Δ*sodA* dissemination was attenuated compared with WT (Fig. 5G). This provides

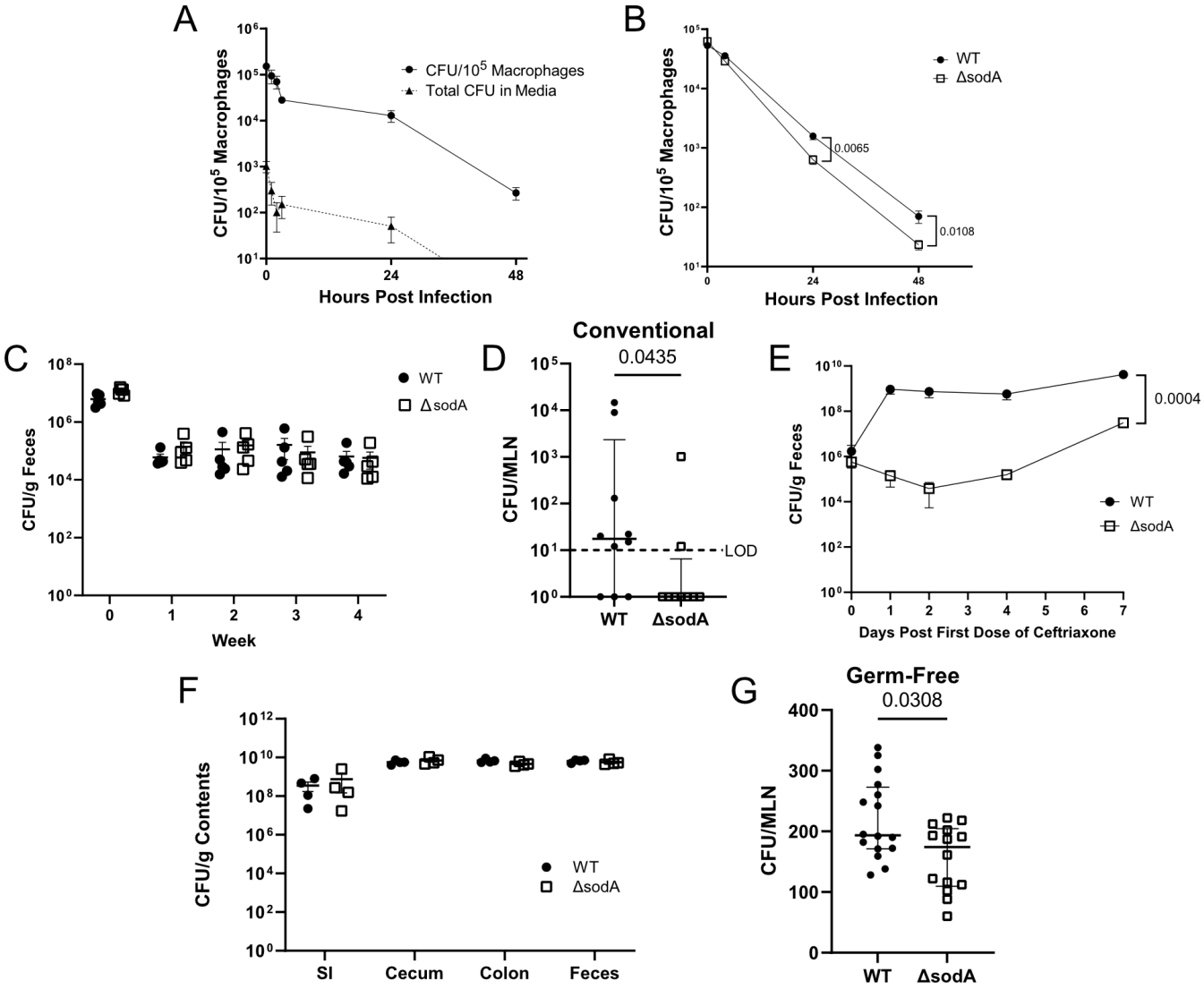

FIG 5 Intracellular *E. faecalis* survival supports dissemination to the MLN. (A) J774.A1 macrophages were co-cultured with *E. faecalis* for 1 hour, and an antibiotic protection assay (150 µg/mL gentamicin, 100 µg/mL vancomycin) was performed to evaluate intracellular survival over time (solid line, circles). Total CFU obtained from culture media was assessed to control for extracellular *E. faecalis* (dashed line, triangles). (B) Intracellular survival of *E. faecalis* that lack MN-dependent superoxide dismutase (ΔsodA) (open squares), compared with OG1RF wild type (WT) (closed circles). (C) Mice were colonized with WT or Δ*sodA* by suspending the bacteria in drinking water for 10 days, and fecal abundance was enumerated weekly as CFU per gram feces. (D and E) Mice were colonized with WT or Δ*sodA* and treated with ceftriaxone as in Fig. 1A. MLNs were harvested to enumerate *E. faecalis* dissemination (D), and feces was collected throughout the experiment to track intestinal *E. faecalis* abundance (E). (F and G) Germ-free mice were colonized as in Fig. 1D. (F) Contents from the SI, cecum, colon, and feces were collected to enumerate colonization. (G) MLNs were harvested to enumerate *E. faecalis* dissemination. (A and B) Data are pooled from two independent experiments performed in triplicate. Mean and standard error of the mean are reported, and statistical significance was determined using an unpaired *t*-test. (C, E, F) Data are representative of two independent experiments, using five mice per group, and *P* values were calculated using unpaired *t*-tests. (D and G) Median and interquartile range are reported, and *P* values were calculated using a Mann-Whitney test. Data are pooled from three (D) or four (G) independent experiments, using three to five mice per group. LOD, limit of detection (10 CFU/organ); $P > 0.05$ not reported.

additional support for our hypothesis that intracellular survival within colonic macrophages contributes to *E. faecalis* dissemination to the MLNs.

DISCUSSION

Bacteria have developed numerous mechanisms that support their colonization, survival, and pathogenesis within host organisms. To date, no clear consensus exists regarding the mode of bacterial passage through systemic circulation. Planktonic, free-floating

bacteria within the blood or lymph are immediately opsonized by immunoglobulins and complement proteins that immobilize, perforate, and target invading bacteria for phagocytic uptake. Thus, the development of strategies that support intracellular survival within migratory cells provides bacteria with protection from the hostile environment of the circulatory system. In this study, we investigated how intracellular survival of the commensal pathobiont, *E. faecalis*, contributes to its dissemination during dysbiosis. Despite previous evidence that *E. faecalis* effectively survive intracellularly (19, 27–30), no studies have been conducted to test whether this contributes to *E. faecalis* dissemination from the gut to extraintestinal sites. In this study, we leveraged the intracellular survival-deficient *E. faecalis* strain, *ΔsodA* to show, for the first time, that intracellular survival contributes to *E. faecalis* escape from the gut and dissemination to the MLNs. These data provide support for our hypothesis that intracellular survival within migratory APCs is a mechanism by which *E. faecalis* disseminate to the MLNs (Fig. 6).

Bacterial dissemination often occurs during states of intestinal inflammation or dysbiosis (2, 12, 17, 24, 33). Various mechanisms have been described that explain how bacteria translocate across the intestinal epithelium (9–12, 49, 50) (Fig. 6A); however, our understanding of the processes that contribute to the subsequent dissemination of bacteria to the MLNs and other extraintestinal organs remains unclear. Here, we sought to examine the role that phagocytic APCs play in *E. faecalis* dissemination (Fig. 6B).

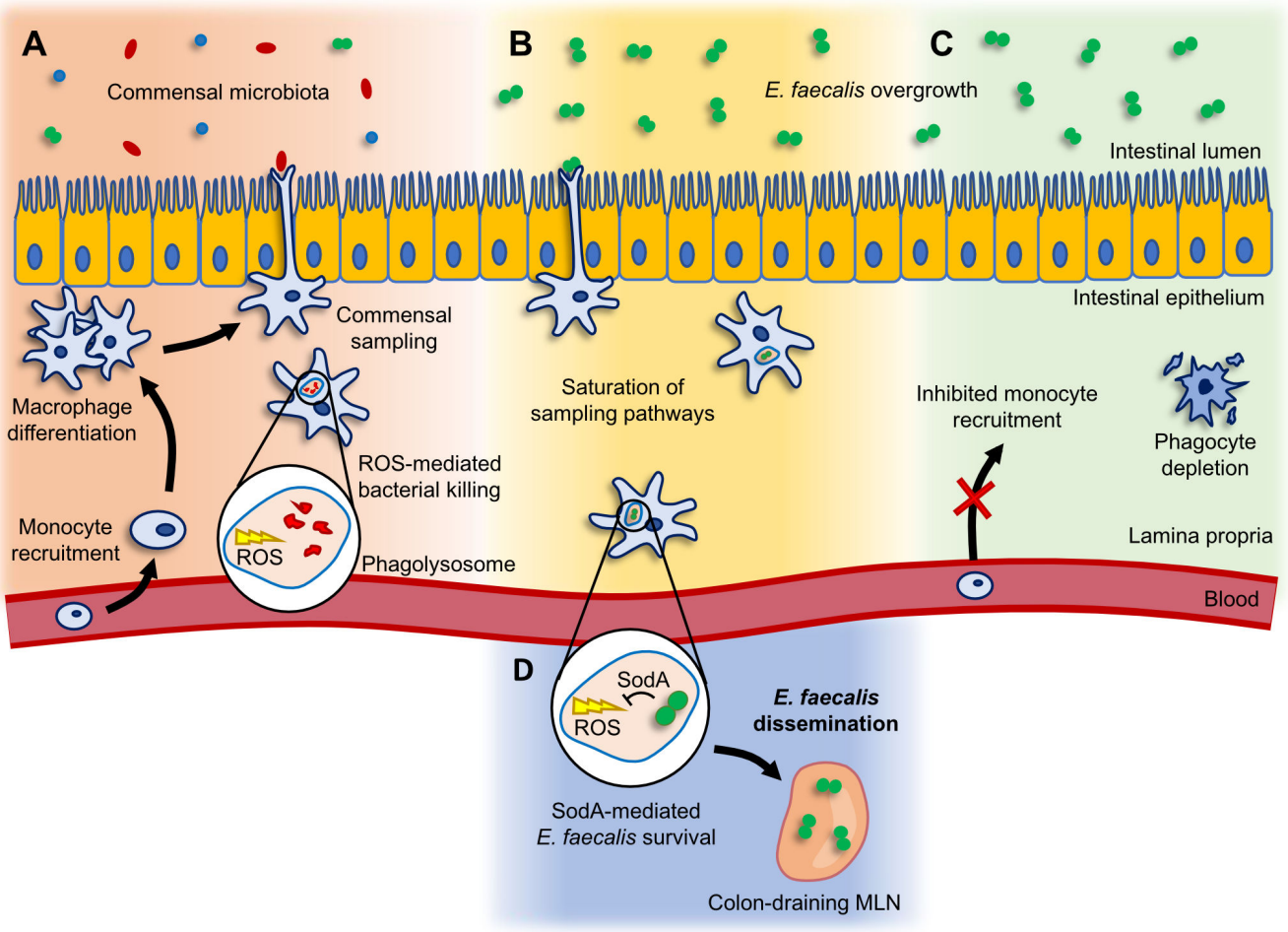

FIG 6 Working model for phagocyte-mediated *E. faecalis* dissemination. (A) Monocytes are recruited from the blood and develop into macrophages that sample luminal bacteria during homeostatic conditions. (B) Upon antibiotic-induced *E. faecalis* overgrowth in the colon, the commensal sampling pathways become saturated by *E. faecalis*. (C) Disruption of monocyte recruitment or depletion of phagocytes ablates the commensal sampling processes and prevents *E. faecalis* translocation across the epithelial barrier. (D) SodA-mediated intracellular survival allows *E. faecalis* to disseminate to the colon-draining MLN.

We found that global phagocyte depletion led to reduced *E. faecalis* dissemination to the MLNs, suggesting that APCs play a role in promoting *E. faecalis* escape from the gut. More specifically, we demonstrated that monocyte-derived CX3CR1+ macrophages are involved in *E. faecalis* dissemination during ceftriaxone treatment (Fig. 6C). Surprisingly, we found that the migration of CD11c+ and CD103+ *bona fide* DCs to the MLN did not contribute to *E. faecalis* dissemination. It is important to note that the treatment regimens used to manipulate phagocytes in this study do not completely ablate phagocytes or their migration. Furthermore, the differences in drug administration route, frequency, timing, and concentration should also be taken into consideration, as these variables likely influence the viability and migration of distinct APC subsets. Nonetheless, our observations raise questions regarding alternative mechanisms of APC migration and subsequent microbial trafficking that have yet to be described.

Although it is accepted that CD103+ APCs are *bona fide* migratory DCs within the GALT, controversy surrounds the migratory potential of CX3CR1-expressing cells. Some researchers have suggested that CX3CR1-int APCs of the GALT should also be considered *bona fide* DCs, as they were shown to descend from DC progenitors, effectively prime naïve T cells, and continuously migrate in lymph (35, 51). In contrast, others have shown that CX3CR1-int APCs are monocyte derived but support the claims that they are migratory (52, 53). These studies also explicitly classify CX3CR1-hi APCs as non-migratory, tissue-resident macrophages. A separate study contradicts this assertion, showing that, during *Salmonella* infection, CX3CR1-hi APCs could be obtained from afferent lymphatics draining to the MLNs, suggesting that these cells do have the capacity to migrate (24). Importantly, these studies were conducted using disparate models of intestinal inflammation and antibiotic-induced dysbiosis, suggesting that their observations may be context dependent. Furthermore, their differential use of CD11b and CD11c to analyze specific subpopulations of APCs highlights the complexity of the migratory APC system in the GALT and the need for better characterization of intestinal APC subsets. Our findings that monocyte-derived macrophages contribute to *E. faecalis* dissemination may provide support for the stance that CX3CR1-expressing APCs are migratory. However, more direct experimentation would be required to confirm whether these cells traffic to the MLN during ceftriaxone-induced dysbiosis or whether their involvement in *E. faecalis* dissemination is the result of sampling at the epithelial interface.

Our study also provides insight into the poorly understood dynamics of commensal dissemination. Our observation that *E. faecalis* dissemination occurs in GF mice in the absence of antibiotic treatment supports previous reports that *Enterococcus* dissemination is the consequence of its overabundance in the gut (2, 33). We propose that *E. faecalis* dissemination is the result of homeostatic commensal sampling mechanisms performed by the host, which become saturated upon expansion of *E. faecalis* populations (Fig. 6B). As such, we expect that low levels of *E. faecalis* dissemination occurs at a steady state, albeit to a degree that is undetectable by methods of culturing or 16S sequencing. During states of dysbiosis, however, when *E. faecalis* is overrepresented in the gut, disseminating *E. faecalis* become readily detectable. We hypothesize that this is a general phenomenon of commensal bacteria and is not restricted to *E. faecalis*. We also expect bacterial taxa that are proficient in intracellular survival to be more likely to disseminate and persist within extraintestinal organs following translocation across the gut epithelium (Fig. 6D).

Mounting evidence suggests that the presence of live bacteria within organized lymphatic tissues may serve homeostatic functions by modulating B cell activity. For instance, commensal *Alcaligenes* spp. induce the production of antigen-specific IgA, which aids their stable colonization of Peyer's patches (54). This cooperation provides a unique and preferred niche for *Alcaligenes* while offering host protection through IgA production. DC-mediated commensal delivery to the MLN also promotes the induction of IgA (16), and the presence of Gram-negative commensal bacteria in MLNs can elicit the production of IgG which confers cross-reactive protection against

infection by pathogens like *Salmonella* (17). These studies suggest that the host may permit controlled colonization of secondary lymphoid organs in the GALT to modulate homeostatic humoral immunity and protect against disease. We propose that antibiotic-induced *E. faecalis* dissemination is the consequence of these permissive systems and that monocyte-derived CX3CR1-expressing cells are important arbiters of these processes. Currently, we cannot definitively conclude whether macrophage-mediated *E. faecalis* dissemination is the result of coordinated translocation across the epithelium, the direct transport of *E. faecalis* to the MLN, or both. Indeed, CX3CR1+ cells have the capacity to extend transepithelial dendrites that facilitate bacterial translocation across the epithelium (9). Furthermore, CX3CR1-hi APCs have been implicated in trafficking bacteria directly to the MLN in a CCR7-dependent manner (24). However, our data indicate that CCR7 is dispensable for this process, suggesting that CX3CR1-expressing APCs may rely on another receptor for chemotaxis to the MLN. Future studies should investigate the milieu of migratory APC subsets that may be responsible for bacterial transport, placing emphasis on the chemokine receptors that guide them.

Here, we provided evidence that monocyte-derived macrophages facilitate the dissemination of a common commensal pathobiont, *E. faecalis*. We showed that this dissemination is mostly restricted to the colon-draining MLN and likely occurs during states of *E. faecalis* overgrowth. We also clearly show that intracellular *E. faecalis* survival is crucial for its dissemination to the MLN. This study builds on a growing body of evidence that implicates migratory host cells in the delivery of live bacteria to the MLNs. While these mechanisms likely support homeostatic maintenance within the GALT, they can also be exploited by intracellular pathogens to gain access to extraintestinal organs and cause disease. We expect that many enterococcal infections originate in this way, particularly in immunocompromised hosts, or when intestinal homeostasis is disrupted by antibiotics. Our work suggests that inhibition of macrophage recruitment in the context of antibiotic therapy could reduce the risk of opportunistic infections by limiting extraintestinal escape of pathobionts like enterococci. Improving our understanding of the migratory APC network within the GALT will likely shed light on other strategies for preventing or treating infections by disseminated enteric pathogens.

MATERIALS AND METHODS

Animals

Five- to 6-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (room RB08, JAXWEST facility, Sacramento, CA). Mice were acclimatized for 1 week prior to conducting experiments. Mice were fed a standard chow diet (PicoLab laboratory rodent diet) and de-chlorinated reverse osmosis water *ad libitum*. All mice were maintained under specific pathogen-free conditions throughout the course of the experiments, and mice that were colonized by experimental *E. faecalis* strains were housed in an ABSL2 facility. Five- to 10-week-old male and female germ-free mice were obtained from an in-house breeding colony and housed using the Innorack IVC Mouse 3.5 and Innovive IVC Rodent Caging System (Innovive) that allow isolation and colonization of groups of mice while otherwise maintaining gnotobiotic status. Germ-free mice were maintained on sterilized 2020SX Teklad Global Soy Protein-Free Extruded Rodent Diet (Envigo) and provided autoclaved Milli-Q water (IQ 7000, MilliporeSigma) *ad libitum*. All mice were humanely euthanized by CO_2 asphyxiation followed by cervical dislocation.

Bacterial strains

Laboratory strains of *E. faecalis* used in this study are as follows: OG1RF (spontaneous rifampin-resistant derivative of OG1) (45), CK135 (spontaneous rifampin-resistant derivative of OG1 that also confers enhanced cephalosporin resistance) (55), and HH29 (Δ*sodA)* (in-frame deletion of the *sodA* locus from OG1RF that confers oxidative stress-sensitivity) (30, 47).

Colonization of mice with *E. faecalis* strains

Cultures of *E. faecalis* were grown to stationary phase overnight in Mueller-Hinton broth (Difco), supplemented with 50 µg/mL rifampicin (Chem-Impex), at 37°C, and shaking at 200 rpm. Cultures were pelleted by centrifugation at 4,000 relative cetrifugal force (rcf) for 10 minutes, and the pellets were washed with autoclaved Milli-Q water two times to remove excess media and antibiotics. Final pellets were resuspended in autoclaved Milli-Q water, and bacterial cell concentration was determined by measuring the optical density at 600 nm with a spectrophotometer (NanoDrop 2000 spectrophotometer; Thermo Scientific). Colonization was performed as described previously (2, 32). Briefly, mice were fed 5×10^8 CFU/mL of bacteria in 300 mL of drinking water which was replaced every 3–4 days. For conventional mouse colonization experiments, *E. faecalis*-containing water was removed and replaced by normal drinking water 10 days following the start of feeding. For germ-free mouse experiments, mice were maintained on *E. faecalis*-containing water for 7 days prior to euthanasia.

Ceftriaxone treatment

Mice were maintained on *E. faecalis*-free drinking water for 3 days prior to performing injections. All injections were performed intraperitoneally (IP) using insulin syringes (BD Biosciences). Ceftriaxone (ceftriaxone for injection USP; Apotex Corp.) was suspended in saline (0.9% sterile-preservative free; Phoenix) to generate a 25 mg/mL solution. Mice were injected with 100 µL ceftriaxone solution (2.5 mg/injection) once daily for 5 consecutive days. Control mice received equal volumes of sterile saline. Mice were euthanized 3 days following the final ceftriaxone injection.

Intracolonic clodronate liposome administration

Prior to intracolonic administration, mice were anesthetized by IP injection of 100 µL ketamine/xylazine solution (12 mg/mL ketamine, 1.6 mg/mL xylazine). Once anesthetized, mice were placed on a warm heating pad covered with clean dressing. One hundred microliters of clodronate-containing liposomes or control liposomes (Liposoma) was administered by inserting a steel blunt-tipped gavage needle (small-bore) approximately 3 cm into the rectum. Mice were returned to their respective cage which was maintained on a heat pad until all mice recovered from anesthesia. Intracolonic liposome administration was performed on the final day of *E. faecalis* colonization and on the final day of ceftriaxone treatment (7 days apart). Mice received no more than 200 µL of IP-injected volume per day.

CCR7 blockade

CCR7 blockade was adapted from previous studies (56). Briefly, anti-CCR7 monoclonal antibodies (αCCR7) (eBiosciences) were suspended to 5 mg/mL in sterile phosphate-buffered saline (PBS) (pH 7.4). Mice were injected with 100 µL of αCCR7 solution every other day starting on the first day of ceftriaxone treatment (four total injections). Control mice received equal volumes of rat IgG2a kappa isotype control antibody (eBiosciences) suspended to 5 mg/mL in sterile PBS (pH 7.4). Mice received no more than 200 µL of injected volume per day.

CCR2 antagonism

CCR2 antagonism was performed using the chemical antagonist RS 102895 hydrochloride (CCR2-a) (Tocris Bioscience) (44). CCR2-a was suspended in dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific) and diluted to 2 mg/mL in sterile saline (10% DMSO). Mice received 100 µL IP injections of CCR2-a daily for 7 consecutive days (prepared fresh each day). Daily injections were needed to sufficiently reduce macrophage populations, consistent with the short half-life of RS102895 (57). Control mice received equal volumes of 10% DMSO in sterile saline. For experiments where CCR2-a and ceftriaxone were both

administered, CCR2-a treatments began on the first day of ceftriaxone injection and continued as described until the day of euthanasia. Mice received no more than 200 µL of injected volume per day.

Tissue collection for enumeration of *E. faecalis* colonization and dissemination

E. faecalis was enumerated from fresh fecal samples or representative intestinal samples by dilution plating on brain-heart infusion (BHI) agar (BD Biosciences), supplemented with 200 µg/mL rifampicin, and incubated at 37°C overnight to enumerate *E. faecalis* CFU/g sample. To enumerate *E. faecalis* dissemination, MLNs were sterilely collected in 500 µL ice-cold PBS (pH 7.4). MLNs were homogenized using a tissue homogenizer (Omni-International, TH-115), and the homogenate was plated on BHI agar, supplemented with 200 µg/mL rifampicin, using sterile glass plating beads, and incubated at 37°C overnight to enumerate *E. faecalis* CFU/organ.

In vitro J774 macrophage co-culture with *E. faecalis*

J774A.1 macrophages (ATCC) were cultured at 37°C, 5% CO_2 in 100-mm tissue culture-treated petri dishes (CELLTREAT) containing 10 mL of filter-sterilized feed medium [Dulbecco's modified Eagle medium (DMEM) containing 2 mM glutamine (Gibco) and 10% FBS (Premium, Bio-Techne) and supplemented with penicillin-streptomycin (1×) (Gibco)], replacing media as needed (~every 4 days). Prior to performing co-culture experiments, cell lines were passaged a minimum of three times. Adherent cells were lifted using cell scrapers (Sarstedt) before transferring to a fresh petri dish containing 10 mL feed medium. For co-culture experiments, ~80% confluent macrophage cultures were washed with Dulbecco's phosphate-buffered saline (DPBS) as before and harvested using cell scrapers. Cell suspensions were pelleted by centrifugation at 600 rcf and counted via hemocytometer. 10^5 macrophages were seeded into 10-mm, 12-well tissue culture-treated plates (Corning) containing 1 mL of feed medium and allowed to adhere for 16 hours. *E. faecalis* strains were cultured and washed as described above and suspended to 10^6 CFU/mL in antibiotic-free feed medium. The antibiotic-containing media were aspirated from each well, and the macrophages were gently washed twice with DPBS before 1 mL of *E. faecalis*-containing media (10^6 CFU, MOI = 10) was added to each well. The co-culture plates were centrifuged at room temperature for 5 minutes at 600 rcf to enhance *E. faecalis* contact with adherent macrophages before incubating at 37°C, 5% CO_2 for 1 hour. The *E. faecalis*-containing media were then removed, and macrophages were washed three times with DPBS before adding 1 mL of feed medium containing 150 µg/mL gentamicin and 100 µg/mL vancomycin to rapidly eliminate extracellular *E. faecalis*. Cultures were maintained at 37°C, 5% CO_2 prior to each timepoint where the media from respective wells were collected and plated on BHI agar supplemented with 200 µg/mL rifampicin to enumerate extracellular *E. faecalis* growth. Macrophages were then washed twice with DPBS and lysed with 500 µL of saponin solution (10 µg/mL saponin in DPBS) while scraping with the back end of a pipette tip. Macrophage lysate was pelleted in a microcentrifuge tube at 20,000 rcf for 5 minutes, washed with DPBS, and similarly plated to enumerate intracellular *E. faecalis* CFU.

Cell isolation

Lamina propria

Colons were excised, dissected longitudinally, and the luminal contents were removed by shaking in 50-mL conicals containing PBS and transferred to 10 mL ice cold R10 media [10% FBS, 10 mM HEPES, 1% non-essential amino acids (100×), 1% sodium pyruvate (100×), and 220 µM β-ME (added fresh) in RPMI 1640 + GlutaMAX (1×) (Gibco)]. Tissues were cut into ~2-cm pieces and transferred to a 50-mL conical containing 40 mL HBSS (no Ca, Mg, Phenol Red) containing 10% FBS, 5 mM EDTA, 1 mM DTT, and 10 mM HEPES and incubated at 37°C, shaking at 300 rpm for 30 minutes to remove epithelial cells.

Tissues were washed three times with wash media (10% FBS in DMEM) and mechanically dissociated by mincing thoroughly with a razor blade. Dissociated tissue was transferred to 20 mL of RPMI 1640 + GlutaMAX containing 10% FBS, 0.5 mg/mL Collagenase D (Millipore Sigma), 50 µg/mL DNase I (Worthington), and 25 µg/mL trypsin inhibitor and incubated at 37°C, shaking at 300 rpm for 45 minutes. Tissues were passed through a 100-µm nylon mesh cell strainer (Falcon) while periodically washing with wash media before pelleting cell suspensions at 600 rcf for 5 minutes at 4°C. Cells were passed through a 70-µm nylon mesh cell strainer and pelleted as above before being resuspended in 1 mL FACS buffer (10% FBS in DPBS) containing αCD16/32 Fc block (BD) (1:100). Cells were incubated for 5–10 minutes at room temperature, and 1–10 million cells were transferred to a 96-well U bottom plate (Greiner Bio-One), pelleted at 600 rcf for 10 minutes at 4°C, and resuspended in 100 µL of antibody cocktail in FACS buffer.

Mesenteric lymph nodes

The colonic- and small intestinal-draining MLNs were excised from the mesenteric fat using curved forceps. MLNs from a single mouse were pooled in 3 mL ice cold R10 media. MLNs were mechanically disrupted using the back end of a syringe plunger and passed through a 100-µm nylon mesh cell strainer while periodically washing with 12 mL ice cold R10 media. Cells were pelleted at 600 rcf for 5 minutes at 4°C, resuspended in 1 mL FACS buffer containing αCD16/32 Fc block, and incubated for 5–10 minutes at room temperature. 3×10^6 cells were transferred to a 96-well U bottom plate, pelleted at 600 rcf for 10 minutes at 4°C, and resuspended in 100 µL of antibody cocktail in FACS buffer.

Flow cytometry

FACS buffer containing fluorescence-conjugated antibodies was used to stain single-cell suspensions at 4°C for 30 minutes, unless otherwise stated. The following conjugated antibodies were used: CD3 (17A2), CD19 (6D5), I-Ab (AF6-120.1), CX3CR1 (SA011F11), CD103 (2E7), CCR2 (SA203G11), CCR7 (4B12), CD11b (M1/70), CD11c (N418), Ly6C (HK1.4), CD64 (X54-5/7.1), CD169 (3D6.112), CD14 (M14-23), Tim4 (RMT4-54), and CSF1R (AFS98) from BioLegend. When staining for CCR7, single-cell suspensions were incubated with staining cocktail for 1 hour at 23°C. Following incubation, cells were pelleted at 600 rcf for 5 minutes at 4°C, washed twice with 200 µL of FACS buffer, then resuspended in 200 µL of FACS buffer, and passed through a 30-µm nylon mesh cell strainer (Falcon) into a FACS tube, using 300 µL of FACS buffer to wash the filter (500 µL final volume). Flow cytometry was performed using the BD LSRFortessa (X20), and data were analyzed using FlowJo (V10.8.1) software.

Statistics

Statistical analysis was performed using GraphPad Prism 9, and corresponding statistical methods were reported in the figure legends. Statistical significance for studies that assessed *E. faecalis* dissemination was determined using a Mann-Whitney test. For all other experiments comparing two groups, statistical significance was determined using an unpaired *t*-test. Correlation analysis was performed using a Pearson correlation analysis and simple linear regression.

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ETHICS APPROVAL

All protocols have been approved by the committee for animal care and use at the Medical College of Wisconsin.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (IAI00006-24-s0001.pdf). Figures S1 to S3.

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