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Antibiotics Promote Inflammation Through the Translocation of Native Commensal Colonic Bacteria

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Abstract

Objective—Antibiotic use is associated with an increased risk of developing multiple inflammatory disorders, which in turn are linked to alterations in the intestinal microbiota. How these alterations in the intestinal microbiota translate into an increased risk for inflammatory responses is largely unknown. Here we investigated if and how antibiotics promote inflammation via the translocation of live native gut commensal bacteria.

Design—Oral antibiotics were given to wildtype and induced mutant mouse strains and the effects on bacterial translocation, inflammatory responses, and the susceptibility to colitis were evaluated. The sources of the bacteria and the pathways required for bacterial translocation were evaluated using induced mutant mouse strains, 16s rRNA sequencing to characterize the microbial communities, and *in vivo* and *ex vivo* imaging techniques.

Results—Oral antibiotics induced the translocation of live native commensal bacteria across the colonic epithelium, promoting inflammatory responses, and predisposing to increased disease in response to coincident injury. Bacterial translocation resulted from decreased microbial signals delivered to colonic goblet cells (GCs), was associated with the formation of colonic goblet cell associated antigen passages (GAPs), was abolished when GCs were depleted, and required CX₃CR1⁺ dendritic cells (DCs). Bacterial translocation occurred following a single dose of most antibiotics tested, and the predisposition for increased inflammation was only associated with antibiotics inducing bacterial translocation.

Conclusions—These findings reveal an unexpected outcome of antibiotic therapy and suggest that bacterial translocation as a result of alterations in the intestinal microflora may provide a link between increasing antibiotic use and the increased incidence of inflammatory disorders.

Keywords

Bacterial translocation; Gut immunology; Antibiotics; Inflammation

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Introduction

Antibiotics were introduced into the medical armamentarium less than 100 years ago and altered the course of healthcare and humanity by allowing survival from diseases previously believed to be incurable. Since their introduction, medical antibiotic use has increased dramatically in the United States with a particular increase in the use of broad spectrum antibiotics in recent years¹. In addition, low dose antibiotics have been used for agricultural purposes for over 50 years², leading a nearly ubiquitous exposure to low levels of antibiotics in food and water for individuals who are not receiving antibiotic therapy for therapeutic purposes^{3,4}. Epidemiological studies suggest that this increasing exposure to antibiotics is associated with an increased risk for developing inflammatory diseases including autoimmune disease, inflammatory bowel disease, celiac disease, food allergy, asthma, and obesity⁵⁻¹¹. The spectrum and number of inflammatory disorders associated with antibiotic use and the association of these disorders with a variety of antibiotics suggests that this increased risk is not due to side effects of specific antibiotics, but rather a shared effect across multiple antibiotics impacting a risk for general inflammatory responses. In accordance with this premise, multiple studies have associated alterations in the gut microbiota, including alterations induced by antibiotics, with enhanced inflammatory responses¹²⁻¹⁷. Despite these observations, how dysbiosis of the gut microbial community due to antibiotic therapy confers a risk for enhanced inflammatory responses remains largely unknown.

Disruption of the gut microflora with prolonged courses of oral antibiotics prior to the introduction of pathogens is a commonly used technique in enteric infection models¹⁸⁻²¹. This maneuver provides a niche for the pathogens to occupy within the host, and accordingly antibiotic treatment is closely linked to pathology and dissemination of the enteric pathogens in these models^{18,19}. Recent studies revealed that prolonged antibiotic treatment to disrupt the gut microbiota also resulted in the translocation of gavaged, laboratory adapted, non-pathogenic bacterial species^{22,23}, indicating that bacterial pathogenicity may not be required for epithelial translocation. Whether antibiotic induced translocation applies to native gut commensals, which may not share properties with laboratory adapted bacterial strains and which would be suppressed by antibiotic therapy, is unknown. However, these observations raise the possibility that antibiotic therapy might induce the translocation of native commensal gut species providing an unappreciated link between antibiotic therapy, enhanced inflammatory responses, and untoward outcomes associated with antibiotic therapy. To this end we investigated whether and how antibiotic therapy induces the translocation of native gut commensal bacteria and the outcomes associated with antibiotic induced gut commensal translocation.

Materials and Methods

See supporting supplementary information for details.

Mice

All mice were maintained on the C57BL/6 background and bred in house. Mice were fed routine chow diet and given filtered autoclaved water. Animal procedures and protocols were

carried out in accordance with the institutional review board at Washington University School of Medicine. See supplemental table S2 for specifics on mouse strains and their use in this study.

Antibiotic Treatment

For continual antibiotic treatment mice were treated with a combination of antibiotics (ABX): ampicillin (1 g/L), metronidazole (1 g/L), neomycin (1 g/L), and vancomycin (500 mg/L) in drinking water for 14 days. For one time dosing of a combination of antibiotics, mice were gavaged once with 500µg ampicillin, 500µg metronidazole, 500µg neomycin, and 250µg vancomycin. For one time dosing of single antibiotics, mice were gavaged once with 500µg of ampicillin, streptomycin, metronidazole, ciprofloxacin, erythromycin, neomycin, or tetracycline, or 250µg vancomycin, or 1mg amoxicillin. For sub-therapeutic antibiotic therapy, mice were given vancomycin 6µg/ml in drinking water as previously described⁵.

Statistical Analysis

A student's *t* test, a one way ANOVA with Dunnett's multiple comparisons, or a Mann-Whitney test was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Analysis of the microbial communities was performed with Partek Genomics Suite (Partek Inc., St. Louis, MO).

Results

Antibiotic therapy induces translocation of live colonic commensal bacteria to the mesenteric lymph node (MLN) and induces an inflammatory response

Four days following the administration of a single dose of a combination of oral antibiotics (ABX) to disrupt the gut microbiota, we observed an increase in the inflammatory cytokines CXCL1, IL-17, and IFN γ , but not in the immunomodulatory cytokines TGF β or IL-10 (Fig. 1A). This inflammatory response was accompanied by the translocation of live bacteria to the MLNs in antibiotic treated mice, but not in vehicle treated controls (Fig. 1B), however we did not observe an increase in the size of the MLNs following antibiotic treatment (not shown). Mass spectrometry analysis revealed the bacteria cultured from the MLN following antibiotic treatment were *Enterococcus faecalis* and *Escherichia coli*. While culture based methods may not identify all organisms present within the MLN, these species are consistent with an origin in the gut microbiota (Supplementary Fig. 1). To better identify where the bacteria were translocating within the gastrointestinal (GI) tract, we used a metagenomic approach to characterize the microbial communities present in the small intestine (SI), colon, and MLNs following antibiotic treatment. Principal component analysis of the species identified by bacterial 16s rRNA sequencing four days following ABX treatment revealed that the microbial communities from each organ from multiple mice clustered together (Fig. 1C), while a dendrogram generated by unsupervised hierarchical clustering of bacterial species present in each tissue revealed that the bacteria within the MLNs more closely resembled the colonic community as opposed to that in the SI (Fig. 1D). Likewise a Venn diagram of bacterial species that were represented at least once within each respective tissue in four different mice following antibiotic treatment revealed substantial overlap between the species present within the MLNs with those present in the colon, including species shared

exclusively between the MLNs and colon, but not the SI. Conversely we found no species were shared between the MLNs and SI but not the colon (Fig. 1E). Some species were observed to be present in the MLN but not either the SI or colon, which may be due to the increased depth of sequencing of the MLN when compared with the colon, relative to the diversity of the bacterial community in each organ. Likewise, analysis of the species by the percentage of operational taxonomic units in the major phyla showed a significant difference between the MLN and the SI, but not the MLN and the colon (not shown). The SI and colon predominantly drain into distinct MLNs, a colonic MLN (cMLN) and a SI MLN, which can be identified following the injection of dye into the colonic or SI submucosa (Fig. 1F). Following treatment with antibiotics, we observed live bacteria could be found in the cMLN and to a much lesser extent the SI MLN, while bacteria were not detected in either MLN in untreated mice (Fig. 1G). Therefore, following antibiotic treatment live commensal bacteria translocate across the colonic epithelium to the MLN resulting in an inflammatory response.

Live commensal bacteria translocation is associated with colonic GCs and GAPs

Dendritic cells (DCs) can extend dendrites through the intestinal epithelial layer to sample luminal contents²⁴⁻²⁶. This process of DC trans-epithelial dendrite (TED) extension has largely been observed in the SI as opposed to the colon^{24,25}. *In vivo* imaging of the colon of CD11c^{YFP} and CX₃CR1^{GFP} reporter mice at a time point in which bacterial translocation occurs following treatment with antibiotics did not reveal the presence of TED extension by DCs (Supplementary Fig. 2). In addition we could not find evidence of TED formation by colonic DCs using higher resolution imaging on fixed tissue sections of colon following antibiotic treatment (Fig. 2A and Supplementary movie 1). Therefore, the induction of TEDs by DCs following antibiotic treatment appeared to be an unlikely explanation for commensal bacterial translocation across the colonic epithelium. Goblet cells (GCs) have been reported to act as passages to deliver luminal substances across the intestinal epithelium to DCs²⁷. Goblet cell associated antigen passages (GAPs), were present in the SI, but not the colon at the steady state, and SI GAPs were reported to deliver small soluble antigens, but not inert particles as small as 20nm. However it was recently reported that microbial sensing by colonic GCs suppressed GAP formation²⁸, and that colonic GAPs formed when the microbial load was reduced by antibiotic treatment²⁸ and Supplementary Fig. 2). Mouse atonal homologue 1 (Math1) is a transcription factor required for the development of secretory epithelial cell lineages, which is largely restricted to GCs in the colon (Supplementary Fig. 3). We observed that following antibiotic treatment, bacteria were not present in the cMLN of mice in which Math1 expressing epithelial cells had been deleted (Figure 2B), suggesting that colonic GCs and GAPs might play a role in translocating commensal bacteria. Further we observed fluorescently labeled *Enterococcus faecalis*, which was isolated from the cMLN following antibiotic treatment, (Supplementary Fig. 1), preferentially associated with colonic GCs that formed GAPs, which were identified as epithelial cells with a GC morphology acquiring luminal 10kD dextran, in antibiotic treated mice (Supplementary Fig. 2 and 4). The labeled *Enterococcus faecalis* could be observed within the apical mucin compartment of GCs that had formed GAPs, and near the basolateral surface or within GCs following antibiotic treatment (Fig. 2C and D). To further evaluate the relationship between GCs and GAPs with commensal bacteria we sorted GCs and other non-GC intestinal epithelial cells (IECs) from the colons of mice following

antibiotic treatment and evaluated the presence of commensal bacteria by culture. Live bacteria were only associated with GCs following antibiotic treatment, and were rarely found to be associated with IECs (Fig. 2E). Mass spectrometry analysis revealed that the cultured bacteria that were associated with GCs were *Escherichia coli*, which is similar to the live bacteria isolated from the MLN (Supplementary Fig. 1). To better understand the relationship between antibiotic therapy, GAP formation, and bacterial translocation to the MLN, we evaluated the kinetics of GAP formation and bacterial translocation to the cMLN following continuous antibiotic therapy and withdrawal. Colonic GAPs formed within a few days of starting antibiotic treatment in drinking water *ad libitum* and remained open for approximately 5 days following the removal of antibiotics. Live bacteria appeared in the cMLN two days after the withdrawal of antibiotics and remained in the cMLN during the time in which GAPs remained after antibiotic cessation (Fig. 2F). Disruption of GC microbial sensing has been demonstrated to induce colonic GAPs independent of antibiotic therapy in specific pathogen free housed mice²⁸. We observed deletion of myeloid differentiation primary response gene 88 (Myd88) globally or selectively in Math1 expressing cells, which disrupts GC microbial sensing and induces colonic but not SI GAPs²⁸, resulted in translocation of bacteria to the MLNs in the absence of antibiotic treatment (Fig. 2G and H), thus alleviating concerns that the translocation was secondary to toxic effects of antibiotics on the epithelium, and demonstrating that loss of Myd88 in Math1 expressing lineages, such as GCs, is sufficient to induce bacterial translocation. Recently it was reported that blockade of epidermal growth factor receptor (EGFR) or the downstream p42/p44 mitogen activated protein kinase (MAPK) relieved the repression of colonic GC microbial sensing, and allowed colonic GAPs to form²⁸. We observed pharmacologic inhibition of EGFR or p42/p44 MAPK, which has no effect on SI GAPs²⁸, also resulted in the translocation of live bacteria to the MLN (Supplementary Fig. 5), further linking bacterial translocation with the formation of colonic GAPs. Therefore translocation of live bacteria across the epithelium to the MLN can occur in the absence of antibiotic therapy when GC microbial sensing is disrupted, consistent with the translocation of live native commensal bacteria to the MLNs following antibiotic withdrawal when the microbiota is rebounding yet still transiently altered enough to allow GCs to form GAPs.

Myd88 dependent and independent microbial signals regulate the transfer of live bacteria to the MLN following antibiotic therapy

Following antibiotic treatment, CX₃CR1⁺ DCs can carry luminally inoculated exogenous pathogens and non-pathogens to the MLN²³. Consistent with this, we observed that following antibiotic treatment, the population of CX₃CR1⁺CD11b⁺CD103⁻ DCs was significantly increased in the cMLN (Fig. 3A and B). Additionally following antibiotic treatment, a substantial proportion of the native commensal bacteria in the MLN were intracellular (Fig. 3C), and culture of sorted cMLN DCs populations isolated after antibiotic treatment revealed that live bacteria were found almost exclusively within the CX₃CR1⁺CD11b⁺CD103⁻ DCs (Fig. 3D). Further we observed translocation of these bacteria to the cMLN was abolished in the absence of CX₃CR1 despite no change in the formation of colonic GAPs in CX₃CR1^{-/-} mice in response to antibiotic therapy (Fig. 3E and F). In contrast to what was previously reported for GAPs in the SI at the steady state, we readily observed CX₃CR1⁺ APCs interacting with colonic GAPs following antibiotic

treatment (Fig. 3G), further supporting the role for CX₃CR1⁺ APCs in acquiring native commensal bacteria and transporting them to the MLN after antibiotic therapy.

Antibiotic treatment, or loss of microbial sensing by the global deletion of Myd88, induced CX₃CR1⁺ DCs to carry gavaged exogenous bacteria to the MLN²³. These observations were interpreted to indicate a role for the microbiota and Myd88 dependent microbial sensing restricting DC trafficking to the MLN²³. Therefore we explored a role for the microbiota in the translocation of bacteria to the MLN, independent of its role in suppressing GAP formation. Similar to the prior studies²³, we observed that following antibiotic treatment the CX₃CR1⁺ MLN DC population, which could also be identified as CD103⁻ CD11b⁺ DCs, was increased, and that this increase was dependent upon CX₃CR1 (Fig. 3H). In addition we observed that this increase was ablated when Math1 expressing epithelial cells were depleted, suggesting the increase in MLN CX₃CR1⁺ DCs is also dependent upon GCs and by extension GAPs (Fig. 3H). This is consistent with the suppression of bacterial transfer to the MLN by the microbiota due to the inhibition of GAP formation and bacterial translocation across the epithelium. We evaluated the population of CX₃CR1⁺ DCs in the MLNs of mice where Myd88 deletion was restricted to Math1 expressing lineages to understand if the microbiota affected DC trafficking independent of its effect on inhibiting GAP formation. When colonic GAPs were induced by ablating Myd88 dependent, GC intrinsic, microbial sensing, the CD103⁻ CD11b⁺, or CX₃CR1⁺, cMLN APC population expanded (Fig. 3I), indicating that loss of Myd88 signals in Math1 expressing cells was sufficient to induce DC trafficking to the MLN. Interestingly, we found this cMLN DC population was also expanded in mice globally lacking Myd88 and that continuous antibiotic treatment reduced this DC population in mice lacking Myd88 globally, and in mice lacking Myd88 in Math1 expressing lineages (Fig. 3I). Thus Myd88 dependent microbial sensing in Math1 expressing cells inhibits translocation of bacteria across the colonic epithelium, and non- Myd88 microbial mediated events induce CD103⁻ CD11b⁺ CX₃CR1⁺ DCs to traffic to the MLN.

The enhanced inflammatory responses following antibiotic therapy require GCs and are associated with GAP formation

The presence of bacteria and the associated inflammatory environment we observed within the MLN could result in a predisposition to enhanced and potentially harmful responses to a coincident inflammatory stimuli. Consistent with this, mice with disrupted GC intrinsic microbial sensing, which results in colonic GAP formation and translocation of commensal bacteria to the MLN, had more severe weight loss and disease in the dextran sodium sulfate (DSS) colitis model when compared with their littermate controls (Fig. 4A).

Epithelial recognition of the commensal microbiota plays a role in protection from damage and in epithelial repair²⁹. To reveal these effects, studies used continual treatment with a combination of antibiotics, which would mask effects arising from bacterial translocation, as translocation occurs following antibiotic withdrawal (Fig. 2F), and therefore the detrimental effects of microbial translocation would not be apparent in these studies. We observed the kinetics of colonic GAP formation and bacterial translocation following a single ABX treatment were similar to those found after withdrawal of prolonged antibiotic treatment,

with GAPs forming on day one and bacteria appearing in the MLN on day two (Fig. 4B). This suggests the period of time following antibiotic therapy in which the gut microbiota is rebounding, but is not yet sufficient to suppress colonic GAP formation, may be a vulnerable time for the development of enhanced inflammatory responses to coincident injury. We observed that mice given ABX treatment and DSS on day 0, to allow translocation of bacteria to occur during epithelial damage, lost more weight (Fig. 4C), and had worse pathology (Fig. 4D), with areas of large ulceration and lymphocyte infiltration into the muscle layer when compared with those given DSS alone (Fig. 4E). Antibiotic treatment did not worsen DSS colitis in *Math1^{fl} v^{fl}* mice, which lack GCs and GAPs, consistent with the increased severity of colitis seen after ABX treatment being dependent upon GCs and by extension GAPs, and bacterial translocation (Fig. 4F and G).

Enhanced inflammatory responses only occur following administration of antibiotics that induce GAPs and bacterial translocation

While general antibiotic use has been associated with an increased risk of developing inflammatory diseases, certain classes of antibiotics have been found to have stronger correlations with the disease risk, while others were not associated with an increased risk for disease⁸³⁰. We evaluated the effects of multiple commonly used oral antibiotics, with differing mechanisms of action and spectra (Table S3), at single clinically relevant doses on the induction of colonic GAPs, the translocation of bacteria to the MLN, the production of inflammatory mediators, and on the course of colitis. Bacteria could be found in the cMLN following a single dose of six of the nine antibiotics tested, and this correlated with the induction of colonic GAPs in each case (Fig. 5A and B). The lack of colonic GAP induction or bacterial translocation was not directly correlated with levels of the colonic bacterial load as measured by quantitative PCR for bacteria 16s rRNA (Supplementary Fig. 7), suggesting that the specificity in the bacterial species depleted by the various antibiotics may affect GAP induction and bacterial translocation. In addition, local inflammatory responses, evidenced by significantly increased levels of CXCL1 with slight increases in IL17 and IFN γ in the cMLN were only seen in mice treated with single doses of antibiotics that induce bacterial translocation (Fig. 5C). Similar to the observations with mixed antibiotics, we did not see increased production of immunomodulatory cytokines following single antibiotic doses (Supplementary Figure 7B), indicating that these antibiotic treatments were not globally inducing cytokine production. We observed dissemination of bacteria to distant sites following antibiotic therapy (Supplementary Fig. 6). Accordingly only mice treated with antibiotics that induced GAPs and bacterial translocation had systemic inflammatory responses as seen by significantly increased serum IL6 (Fig. 5C, Supplementary Fig. 7). Moreover when single doses of antibiotics that do and don't promote GAP induction and bacterial translocation were given coincident with DSS induced epithelial damage, only antibiotics inducing GAP formation and bacterial translocation resulted in worsened disease (Fig. 5D and E, Supplementary Fig. 7). Antibiotics given at low doses have been used to promote the growth of farm animals and changes in the gut microbiome due to sub-therapeutic antibiotic therapy has been causally linked to obesity⁵, which has recently been appreciated as an inflammatory disorder³¹. We observed colonic GAP formation and bacterial translocation occurred during continuous sub-therapeutic antibiotic therapy (Supplementary Fig. 1 and 8), suggesting that in addition to the alteration in the gut

microbial community and its metabolic function⁵³²³³, bacterial translocation may provide an additional inflammatory stimulus promoting obesity.

Discussion

Here we have identified a surprising and unappreciated effect of antibiotic use that results in the translocation of live indigenous commensal gut bacteria across the colonic epithelium, resulting in an inflammatory response, and a predisposition to worsened disease in response to coincident injury (Summary Fig. 6). Confocal imaging and analysis of isolated GCs demonstrated that commensal bacteria were only associated with colonic GCs and within colonic GCs after antibiotic treatment. Further we observed that antibiotic induced translocation of gut commensal bacteria was ablated when GCs were absent and translocation of gut commensal correlated with the presence of colonic GAPs. Consistent with our findings a recent study reported that translocation of exogenous bacteria following antibiotic treatment was independent of disruption of the epithelial barrier or alterations in tight junction permeability²². While our observations suggest that gut commensals may use GAPs to traverse the epithelium following antibiotic treatment, the specific mechanism and pathways by which commensal gut bacteria traverse the colonic epithelial layer remain to be explored. GAPs in the SI were unable to transport inert particles as small as 20nm²⁷, and very few CFUs were found in the SI draining MLN in any of our models, despite continuous GAP formation in the SI, yet here colonic GAPs are associated with the translocation of live native commensal bacteria to colon draining MLN. What accounts for the disparity in these observations is unclear, but may be related to differences in the properties of the SI and colonic GCs as well as differences in the properties and density of the bacteria between the SI and colon, as enteric pathogens have been reported to use SI GCs as a portal of entry³⁴.

The microbiota plays dual and reciprocal roles in the process of antibiotic induced commensal bacterial translocation from the intestinal lumen to the MLN. Prior studies demonstrated that GC intrinsic Myd88 dependent microbial sensing inhibits colonic GAP formation²⁸ and the observations here demonstrate that this role for microbial sensing by GCs extends to bacterial translocation, thus defining a role for the gut microbiota in suppressing bacterial transfer across the epithelium. Our observations and prior studies identified that bacterial trafficking to the MLN is dependent upon CX₃CR1⁺ DCs²³. We observed that when Myd88 was depleted from GCs to allow bacteria to cross the epithelium and be acquired by CX₃CR1⁺ DCs, trafficking of these DCs to the MLN was increased, and conversely depletion of the microbiota by antibiotics abolished the increased DC trafficking to the MLN. This affect of the microbiota enhancing DC trafficking to the MLN was Myd88 independent, as these effects were observed in global as well as cell type specific Myd88 knockouts. Thus the microbiota plays dual and opposite roles in the translocation of bacteria to the MLN, first inhibiting bacteria crossing the epithelial layer by inhibiting GAP formation in a Myd88 dependent manner, and second by enhancing CX₃CR1⁺ DC trafficking to the MLN in a Myd88 independent manner after GAPs have formed to expose the underlying DCs to luminal substances.

The dual role of the microbiota in bacterial translocation suggests unexpected relationships between antibiotic use, bacterial translocation, and enhanced inflammatory responses. We

observed that although colonic GAPs were present, bacterial translocation did not occur during continuous high, or therapeutic, doses of antibiotics. This is consistent with continuous therapeutic doses of antibiotics reducing the microbiota to a level that relieves the repression on GAP formation and below the level required to either allow bacteria to cross the epithelium or to deliver the Myd88 independent signals inducing DCs to migrate to the MLN. Bacterial translocation to the MLN occurred several days after the cessation of continuous high dose antibiotics during a window of time in which the gut microbiota had not returned to a level that was sufficient to suppress GAP formation, yet was sufficient to allow bacteria to cross the epithelium and translocate to the MLN. Further consistent with this, we observed that bacterial translocation occurred during continuous low dose sub-therapeutic antibiotic therapy. Thus the most tenuous situations for the translocation of gut bacteria and antibiotic induced inflammatory responses are paradoxically after cessation of therapy or during exposure to low doses of antibiotics. These observations may indicate antibiotic therapy as a contributor to late-onset neonatal sepsis, as a significant proportion of these children are infected with bacterial species that were already present within the gut and these children received more antibiotics prior to septicemia¹⁶. In addition low levels of antibiotics are known to contaminate food and water sources³, suggesting a role for antibiotics in the dramatic increase in obesity and inflammatory diseases that could be independent of intentional antibiotic therapy.

We observed that a single dose of antibiotics was sufficient to cause a dysbiosis of the gut microbiome, which induced GAP formation and bacterial translocation. While bacterial translocation occurred for a limited time until the dysbiosis was corrected in the normal host, in susceptible hosts antibiotic induced alterations in the gut microbiota could be a trigger initiating inflammation, which could maintain the dysbiosis of the gut microbiota thus perpetuating the cycle of bacterial translocation, inflammation, and dysbiosis. This could provide a basis for the observation that prior antibiotic therapy is associated with inflammatory bowel disease, a condition known to be associated with alterations of the gut microbiome^{83035_37}. We evaluated a range of antibiotics with differing spectra and mechanisms of action (Supplementary Table S3). Not all antibiotics, administered at a commonly given dose, resulted in GAP induction and bacterial translocation, however the increased severity of inflammation in response to epithelial injury was only seen with antibiotics inducing GAPs and bacterial translocation. One exception to the dichotomy we observed amongst the antibiotics tested, was ciprofloxacin, which induced GAPs and bacterial translocation, but did not worsen inflammation. This could be related to a previously identified anti-inflammatory property of ciprofloxacin in colitis models³⁸. Interestingly the two bacteriostatic antibiotics tested did not induce GAPs, bacterial translocation, or inflammation, while all antibiotics inducing GAPs, bacterial translocation, and inflammation were bacteriocidal. The antibiotics inducing GAPs, bacterial translocation, and increasing severity of colitis targeted Gram+ species and anaerobes, suggesting that members of Firmicutes, such as *Clostridia*, might be potential candidates regulating GAP formation. However, given the complexity and interdependence of the members of the gut microbiota, it is likely that antibiotics affect gut bacterial species indirectly, and the relevant bacterial species that are altered and regulate GAPs may not be those anticipated by the antibiotic spectra alone. The observation that not all antibiotics induce bacterial translocation

coupled with the observation that those that do induce bacterial translocation can have this effect at very low doses implies that inducing bacterial translocation and resulting inflammation is not solely a property of reducing the global gut bacterial load. Interestingly we observed that a limited number of bacterial species were significantly depleted from the gut microbial community four days after antibiotic therapy when GAPs were present and bacterial translocation to the MLN was high (Supplementary table S5), making these species candidates for regulating this process. Together this gives promise that understanding the interactions between the host and specific members of the gut microbiota that restrict bacterial translocation may be possible and offer new strategies to avoid some of the untoward outcomes related to antibiotic exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GC	goblet cell
GAP	goblet cell associate antigen passage
DC	dendritic cell
ABX	combination of antibiotics (ampicillin, metronidazole, neomycin, vancomycin)
MLN	mesenteric lymph node
cMLN	colonic MLN
GI	gastrointestinal
SI	small intestine
TED	trans-epithelial dendrite
Math1	mouse atonal homologue 1
IEC	intestinal epithelial cell
MAPK	mitogen activated protein kinase

EGFR	epidermal growth factor receptor
APC	antigen presenting cell
Myd88	myeloid differentiation primary response gene 88
DSS	dextran sodium sulfate
CFU	colony forming unit
ELISA	enzyme-linked immunosorbent assay

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Summary Box

What is already known about this subject

Antibiotic use is associated with an increased risk of developing multiple inflammatory disorders. These inflammatory disorders have been linked to alterations in the gut microbiome. How antibiotic induced alterations in the microbiome result in an increased risk for inflammation is largely unknown.

What are the new findings

We observed that following oral antibiotic therapy, indigenous live commensal gut bacteria are translocated across the epithelium to induce inflammatory responses. Bacteria crossing the epithelium resulted from decreased microbial sensing by goblet cells, was dependent upon the presence of goblet cells, and was associated with the formation of goblet cell associated antigen passages in the colon. Most but not all antibiotics tested induced bacterial translocation, and increased inflammation in response to coincident epithelial injury was only associated with antibiotics inducing bacterial translocation.

How might it impact on clinical practice in the foreseeable future?

These findings identify an unexpected affect of antibiotics that increases the risk for inflammation. Understanding the interactions between the host and specific members of the gut microbiota that restrict bacterial translocation may offer new strategies to avoid some of the untoward outcomes related to antibiotic exposure.

Figure 1. Antibiotic therapy induces translocation of live colonic commensal bacteria to the MLN and induces an inflammatory response

(A) Cytokine production measured by enzymelinked immunosorbent assay (ELISA) in MLN four days after vehicle or antibiotic treatment (ABX; single dose of a combination of ampicillin, metronidazole, neomycin, and vancomycin). (B) Quantification of live bacteria by colony forming units (CFUs) cultured from the MLNs of mice treated with vehicle ABX four days earlier. (C) Principal component analysis performed on bacterial species identified by 16s rRNA sequencing from MLN, colon contents, or SI contents of mice treated with ABX four days earlier. (D) Unsupervised hierarchical cluster of bacterial species present in

the SI, colon, and MLN of four mice following ABX treatment. (E) Venn diagram showing overlap of species found on average at least once in each organ from four ABX treated mice. (F) Photomicrographs following subserosal injection of Chicago blue dye into the SI (left panel) or colon (right panel) identifying the MLNs (black boxes), blue dye within the MLN indicates the MLN drains the respective organ (white arrows). (G) Quantification of live bacteria by CFUs cultured from the SI or colon draining MLNs of mice treated with vehicle treated or ABX four days earlier. Data is presented as the mean $\pm$ SEM, * = $p < 0.05$, ABX = antibiotic treatment detailed in methods, $n = 3$ mice per group panel A and F, $n = 4$ mice panels C-E, $n = 10$ mice panels B and G.

Figure 2. Live commensal bacteria translocation is associated with the presence of colonic GCs and GAPs

(A) Confocal image of a single colonic crypt from CX₃CR1^{gfp/+} mice given ABX demonstrating the lack of TEDs (epithelial border denoted by a white dotted line). (B) Quantification of live bacteria by CFUs cultured from the SI or colon draining MLNs of vehicle treated or ABX treated Math1^{i^Δvil} or littermate Math1^{f/f} mice following tamoxifen administration and ABX treatment. (C) Confocal image of a colonic epithelial cells with GC morphology from ABX treated mice given luminal *Enterococcus faecalis* (green) and luminal dextran (red) four hours earlier demonstrating the presence of *Enterococcus faecalis* within the apical mucin compartment of a GC that has taken up dextran, or a GAP (D) Confocal image of colonic tissue from ABX treated Math1^{dtomato} reporter mice given luminal *Enterococcus faecalis* (green) six hours earlier, demonstrating the presence of *Enterococcus faecalis* at the lateral border of a Math1 expressing cells with GC morphology. (E) Quantification of CFUs after plating sorted goblet cells (GCs) or non-GC intestinal epithelial cells (IECs) from ABX or vehicle treated mice. (F) Graphical representation of GAPs per GC and corresponding CFUs per MLN during continuous ABX therapy and withdrawal. (G) GAPs per GC in the SI and colon and (H) CFUs in the MLNs of wildtype, Myd88^{-/-}, and Myd88^{i^ΔMath1} mice following treatment with RU486 to delete Myd88 in Math1 expressing cells. Scale bar in A = 50μm, scale bar in C and D = 20μm. Data is presented as the mean ± SEM, * = p<0.05, ND = not detected, ns = not significant, n = 3 or more mice per group or time point panels A, and C-F. Each symbol represents one mouse panels B and H.

Figure 3. Myd88 dependent and independent microbial signals regulate trafficking of live bacteria to the MLN by CX3CR1⁺ DCs following antibiotic therapy

(A) Flow cytometry plots showing changes in the populations of CD103⁺ CD11b⁻ CX3CR1⁻ and CD103⁻ CD11b⁺ CX3CR1⁺ DCs (CD45⁺ CD11c⁺ MHCII⁺) in the colonic MLN after vehicle or ABX treatment; histogram gated on the populations (right panel) demonstrates that CD11b⁺ CD103⁻ DCs are CX3CR1⁺ and CD11b⁻ CD103⁺ DCs are CX3CR1⁻. (B) Absolute number of CD103⁻ CD11b⁺ CX3CR1⁺ DCs in the colonic MLN after vehicle or ABX treatment. (C) Quantification live bacteria in the MLN of ABX treated mice by CFUs demonstrating the total bacterial load, intracellular bacterial load, and antibiotic resistant bacteria determined as outlined in the methods. (D) Quantification of live bacteria by CFUs in the sorted MLN DCs from mice following vehicle or ABX treatment. (E) Quantification of live bacteria by CFUs in the SI and colonic MLNs from CX3CR1^{-/-} and CX3CR1^{+/-} mice following ABX treatment. (F) Graphical representation of GAPs per goblet cell in wildtype and CX3CR1^{-/-} mice following vehicle or ABX treatment. (G) Confocal image of CX3CR1GFP⁺ cell interacting with a dextran (red) filled colonic GC following ABX therapy. (H) Absolute number of CD103⁻ CD11b⁺ MLN DCs in wildtype mice, Math1^{i⁻vil} mice given tamoxifen to delete GC, or CX3CR1^{-/-} mice following vehicle or ABX treatment. (I) Absolute number of CD103⁻ CD11b⁺ MLN DCs in the MLN of wildtype mice, Myd88^{-/-} mice, or Myd88^{i⁻Math1} mice given RU486 to delete Myd88 given no antibiotics or continual ABX therapy in drinking water. Data is presented as the mean $\pm$ SEM, * = $p < 0.05$, ns = not significant, ABX= antibiotics, n=4 or more mice in each group for data.

Figure 4. Enhanced colitis following antibiotic treatment is associated with Gaps and dependent upon GCs

(A) Weight loss in $Myd88^{\Delta}Math1$ mice given RU486 to delete $Myd88$ in GCs or littermate controls placed on 2% DSS in drinking water. (B) Quantification of Gaps per goblet cell and live bacteria in the MLNs by CFUs after a single gavage ABX on Day 0. (C) Weight loss in mice placed on 2% DSS in drinking water, after single dose of ABX or vehicle on Day 0. (D) Graphical representation of total histology scores and (E) representative images showing increased edema, ulceration (white arrows) and lymphocyte infiltration (black arrows) in DSS treated mice given vehicle or ABX as in C. (F) Weight loss and (G) graphical representation of total histology scores in $Math1^{\Delta}vil$ mice given tamoxifen to delete GCs or littermate controls given ABX on day 0 and 1.5% DSS in drinking water. Data is presented as the mean $\pm$ SEM, * = $p < 0.05$, ns = not significant. Scale bar = 50 μ m. $n = 5$ mice per group panels A-E, $n = 3$ mice per group panel F and G.

Figure 5. Single doses of antibiotics that induce colonic GAPs and bacterial translocation worsen inflammatory responses and colitis in response to coincident epithelial injury

(A) Quantification of live bacteria by CFUs cultured from the MLN and (B) quantification of GAPs per colonic crypt four days after single antibiotic treatment. (C) Quantification of MLN or serum cytokines by ELISA four days after single antibiotic treatment. (D) Weight loss in mice given single antibiotics on day 0 and placed on 2% DSS in drinking water. (E) Graphical representation of total histological scores in mice given single antibiotics on day 0 and given DSS in drinking water. Data is presented as the mean $\pm$ SEM, * = $p < 0.05$, ND = not detected, ns = not significant. $n = 5$ mice per group.

Figure 6. Model of the antibiotic induced bacterial translocation and inflammation

(left panel) In the presence of a normal gut microbiota and intact GC microbial sensing, colonic GAPs and bacterial translocation are inhibited. (middle panel) Following antibiotic therapy the altered microbiota is no longer able to inhibit GAP formation and bacterial translocation across the epithelium resulting in low level inflammation. Coincident epithelial damage leads to increased inflammation and worsened colitis. (right panel) The microbiota has dual and opposite roles in bacterial translocation to distant sites. Myd88 dependent GC sensing of the microbiota inhibits GAP formation and bacterial translocation across the epithelium, while Myd88 independent microbial signals induce APCs to migrate to the MLNs.