Published in final edited form as:

Cancer Res. 2013 July 15; 73(14): 4222–4232. doi:10.1158/0008-5472.CAN-13-0022.

Intestinal bacteria modify lymphoma incidence and latency by affecting systemic inflammatory state, oxidative stress, and leucocyte genotoxicity

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Abstract

Ataxia-telangiectasia (A-T) is a genetic disorder associated with high incidence of B cell lymphoma. Using an A-T mouse model, we compared lymphoma incidence in several isogenic mouse colonies harboring different bacterial communities, finding that intestinal microbiota are a major contributor to disease penetrance and latency, lifespan, molecular oxidative stress and systemic leucocyte genotoxicity. High throughput sequence analysis of rRNA genes identified mucosa-associated bacterial phylotypes that were colony-specific. *Lactobacillus johnsonii*, which was deficient in the more cancer-prone mouse colony, was causally tested for its capacity to confer reduced genotoxicity when restored by short-term oral transfer. This intervention decreased systemic genotoxicity, a response associated with reduced basal leucocytes and the cytokine-mediated inflammatory state, and mechanistically linked to the host cell biology of systemic genotoxicity. Our results suggest that intestinal microbiota are a potentially modifiable trait for translational intervention in individuals at risk for B cell lymphoma, or for other diseases that are driven by genotoxicity or the molecular response to oxidative stress.

Keywords

Ataxia telangiectasia; genotoxicity; *Lactobacillus johnsonii*; lymphoma; oxidative stress

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The authors disclose no potential conflicts of interest.

Introduction

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder associated with high incidence of lymphoid malignancies, neurological degeneration, immunodeficiency, radiation sensitivity and genetic instability (1). Approximately 30-40% of all A-T patients develop neoplasia during their life (2): more than 40% of tumors are non-Hodgkin's B cell lymphomas, about 20% acute lymphocytic leukemias, and 5% Hodgkin's lymphomas (3). A-T is caused by biallelic mutations in the ATM gene. Over 600 different ATM mutations have been described (www.LOVD.nl/ATM). The ATM gene encodes a ~350 kDa protein, phosphatidylinositol-3-like kinase, which is expressed abundantly in multiple tissues (4) and plays an important role in cell cycle checkpoint control as well as repair responses to DNA double strand breaks (DSBs) (5). Absence of functional ATM protein results in chromosomal breakage and rearrangements, aberrant V(D)J recombination, and heightened sensitivity to radiation and chemicals with radiomimetic and prooxidant activity.

Although investigations into A-T have been greatly enhanced by the development of mouse models, disease penetrance in genetically identical mouse colonies at different laboratories can vary widely. Some ATM-deficient (*Atm*^{-/-}) mice develop early lymphomas and have short life spans (2-5 months) (6) while others display dramatically delayed phenotypes, where 50% of the mice remain viable after 7-12 months (7, 8). Lifespan studies on inbred and mixed background mice have failed to show phenotypic differences (9), suggesting other factors besides genetic diversity are contributing to disease penetrance. Environmental factors such as housing conditions and diet have been postulated to be contributing factors (10). In this study, we examined another potential contributor – the intestinal microbiota.

Intestinal bacteria have been implicated in several types of cancer. In animal models of colorectal cancer, lower incidences in germ-free or antibiotic-treated animals point toward intestinal microbes playing a causative role (11, 12). *Helicobacter* species have been associated with enhanced carcinogenesis including liver cancer, colon cancer, and mammary carcinoma (13). Bacterial products have also been associated with increased immune activation and inflammation as well as hyperplasia (14, 15). Conversely, probiotic formulations containing lactic acid bacteria have been shown to reduce the incidence of chemically mediated hepatocellular carcinoma and colon cancer in rats (16).

The objective of this study was to examine the role of intestinal bacteria in the penetrance of lymphoma in *Atm*^{-/-} mice. We first demonstrate the effects of housing and intestinal bacteria on lifespan, lymphoma latency, oxidative stress, and systemic DNA damage (or genotoxicity). Next, high throughput sequence analysis was used to identify mucosa-associated bacteria from animals reared in two distinct housing conditions, differing in intestinal microbiota and the aforementioned ATM-deficient traits. Finally, after determining that *Lactobacillus johnsonii* was higher in abundance in our more cancer-resistant mouse colony, we demonstrated its ability to reduce systemic inflammation and genotoxicity when administered to animals from our more cancer-prone colony.

Materials and Methods

Animal housing and husbandry

Atm^{-/-} mice were obtained by intercrossing *Atm*^{+/-} mice and identified by genotyping (see Supplemental Materials and Methods for additional details). Mice were housed under standard conditions in accordance with the Animal Research Committee at UCLA. Mice were housed under two types of specific pathogen free (SPF) conditions, where either sterile (SPF-S) or non-sterile (SPF-N) food, water, and bedding were employed. *Atm*^{-/-} mice

harboring RM and CM microbiota were created by rederivation as described in Fujiwara *et al.* (17) and by antibiotic treatment (18) followed by orogastric gavage of CM feces, respectively.

Mouse longevity studies

For the longevity studies, *Atm*^{-/-} mice were kept until they developed signs of tumors or became sick according to ARC protocols, at which time they were euthanized, or if non-symptomatic kept until they were found dead. Mice were sent to a veterinary pathologist for necropsy. Differences in longevity and lymphoma latency were analyzed using the log-rank test.

Pun⁺ reversion assay

Pun reversions were counted in mice that were 10 days old. Pun reversions can be seen as a black spot on the fur and were counted as described previously (19). Statistics were done using the χ^2 test.

Oxidative stress

Frozen blood was prepared for enzymatic measurement of the oxidized form of glutathione using the Bioxytech® GSH:GSSG-412 kit assay (Oxis, Beverly Hills, CA, USA). Reduced GSH and GSSG were determined separately by reaction with glutathione reductase. The colorimetric assay was performed in triplicate for each blood sample from all male experimental *Atm*^{-/-} mice. Data was expressed as the ratio of free GSH to GSSG.

Micronucleus assay

Micronuclei were examined in peripheral blood erythrocytes collected from ~6-month old mice and stained with Wright-Giemsa (Sigma-Aldrich, St. Louis, MO, USA). At least 2,000 erythrocytes were counted at 100X magnification as described previously (20). Statistics were done using Student's t-tests.

Alkaline comet assay

DNA strand breaks were measured in peripheral blood cells using the alkaline comet assay. Blood was collected from the facial vein of mice approximately 6-months-old and diluted 1:1 with RPMI + 20% DMSO for storage at -80°C until the assay was performed. The comet assay was basically performed as previously described (20). Statistical analyses were done using Student's t-tests.

Bacteria community analyses of SPF-N and SPF-S mice

Fecal pellets from *Atm*^{+/-} mice (SPF-N and SPF-S) were collected and immediately snap-frozen in liquid nitrogen and stored at -80°C. Nucleic acids from fecal pellets were purified using a phenol-chloroform extraction with bead-beating and a fragment of the 16S rRNA gene targeting the V6-V9 region of most bacteria was amplified with PCR primers 909F (5'-ACTCAAAGGAATWGACGG-3') and 1492R (5'-NTACCTTGTTACGACT-3'). The template was amplified and tagged with a sample-specific 8-nt barcode sequence via a 2-step, low cycle number, barcoded PCR protocol. Pyrosequencing was performed on a GS FLX 454 sequencer at the Norwegian Sequencing Centre. Pyrosequencing reads were quality-filtered using LUCY and clustered into phylotypes at 97% identity using UCLUST. Taxonomic classifications were assigned and alpha and beta diversity metrics were produced using the QIIME software package (21). The VEGAN package was used for permutational multivariate analysis of variance and the indispes package was used to identify phylotypes that were indicators for a colony type; identifying indicators involves analysis of occurrence and abundance. To focus on abundant indicators, we considered only indicators

that had an elevated relative abundance of at least 1% (eg, 0.5% to 1.5%). See Supplemental Materials and Methods for additional details.

Bacteria community analyses of CM and RM mice

Intestinal mucosa samples of CM and RM mice were obtained as described in Presley *et al.* (22). DNA was extracted from these samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), and a 30-second beat-beating step using a Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK, USA). One hundred microliter PCR amplification reactions were performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Inc., Hercules, CA, USA). PCR primers targeted a portion of the 16S and 23S rRNA genes and the hypervariable intergenic region, with the reverse primers including a 12-bp barcode (Tables S1a, b). PCRs contained: 50 mM Tris (pH 8.3), 500 µg/ml bovine serum albumin (BSA), 2.5 mM MgCl₂, 250 µM of each deoxynucleotide triphosphate (dNTP), 400 nM of forward PCR primer, 200 nM of each reverse PCR primer, 4 µl of DNA template, and 2.5 units JumpStart *Taq* DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA). Thermal cycling parameters were 94°C for 5 min; 35 cycles of 94°C for 20 sec, 56°C for 20 sec, and 72°C for 40 sec, and followed by 72°C for 5 min. PCR products were purified using a MinElute 96 UF PCR Purification Kit (Qiagen, Valencia, CA, USA). DNA sequencing was performed using an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA). Clusters were created using a template concentration of 2.5 pM. One hundred base sequencing reads of the 5' end of the amplicons and seven base barcode reads were obtained using the sequencing primers listed in Table S1c. De-multiplexing, quality control, and OTU binning were performed using QIIME (21). OTUs were binned at 97% identity.

Lactobacillus johnsonii isolation and oral inoculation experiments

Lactobacillus johnsonii (strain LJ-RS-1) was isolated from RM wildtype mouse feces using Lactobacillus Selection Agar (BD, Franklin Lakes, NJ, USA). For the oral inoculation experiments, this bacterium was grown on LB agar supplemented with 2% glucose and 0.05% (wt/vol) cysteine at 37°C under anaerobic conditions. The strain was grown overnight and suspended in phosphate-buffered saline (PBS). Prior to inoculation with *L. johnsonii*, CM *Atm*^{-/-} mice were treated with 1 g/L ampicillin (Sigma-Aldrich), neomycin (Thermo Fisher Scientific, Waltham, MA, USA), and metronidazole (Baxter, Deerfield, IL, USA) and 500 mg/L vancomycin (Hospira, Lake Forest, IL) in their drinking water for 1 week as described previously (18). Then, 10⁹ CFU of *L. johnsonii* was administered every other day by orogastric gavage to a group of eight animals for four successive weeks; in addition, the drinking water for same group of animals contained 10⁹ CFU/ml of *L. johnsonii*. Fecal population densities of *L. johnsonii* were measured before, during, and after *L. johnsonii* administration, using a previously described sequence-selective qPCR assay (22). After four weeks, mice were euthanized using 3% isoflurane and analyzed as described below.

Gene expression by RT-PCR

RNA from peripheral blood mononuclear cells (PBMC) or tissue was collected by phenol-chloroform extraction followed by ethanol precipitation. Pellets were resuspended in nucleotide-free water, and RNA was treated with 200 units of DNase I (Ambion, Huntingdon, United Kingdom) for 2 hours at 37°C. RNA levels of transforming growth factor β (TGF-β), interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-10 (IL-10), interferon gamma (IFN-γ), and myeloid differentiation primary response gene 88 (*MYD88*) were determined from cDNA and compared as relative expression changes in *L. johnsonii*-treated animals compared to PBS-treated animals. RT-PCR of gene expression was performed using the TaqMan® Gene Expression Assays system (Applied Biosystems, Carlsbad, CA, USA). The level of gene transcription was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Flow cytometry

PBMC or tissue cell pellets were resuspended in staining buffer. Lymphocytes were stained with CD3, CD4, CD19, and CD335 antibodies (Biolegend, San Diego, California, USA). Cells were fixed in 4% paraformaldehyde-phosphate-buffered saline (PBS) and scanned on a Becton Dickinson FACS Calibur flow cytometer. A minimum of 25,000 events were collected. Flow cytometry data were analyzed using Tree Star FlowJo software. Statistical analyses (Student's *t* test) were used to determine if mean values were different ($P < 0.05$).

Sequence data

DNA sequence data has been deposited at the NCBI Short Read Archive under accession numbers SRA059288 and SRX256360.

Results

Housing affects genetic instability, lifespan, and lymphoma latency of *Atm*^{-/-} mice

When the Schiestl lab moved their mice from Harvard University to the University of California Los Angeles (UCLA) in the year 2000, the median lifespan of their *Atm*^{-/-} mice began to increase over a seven-year period from approximately 4 to 12 months (unpublished observations). To examine the influence of housing conditions on traits associated with ATM deficiency, we first compared *Atm*^{-/-} and wildtype mice from three housing conditions: (i) Harvard housing and (ii and iii) UCLA housing in specific pathogen free (SPF) conditions with either sterile (SPF-S) or non-sterile (SPF-N) food, water and bedding. Genetic instability was assessed using the *p*^{um} reversion assay, which measures DNA deletion events repaired by homologous recombination (19). *Atm*^{-/-} mice at Harvard had a significantly higher level of genetic instability compared to their wildtype littermates (19) (Figure 1a). As lifespan increased in the *Atm*^{-/-} mice over seven years under SPF-S conditions, DNA deletion frequency decreased to 10%. These levels are comparable to the wildtype mice (Figure 1a), indicating loss of the abnormally high genetic instability that has been recognized as a hallmark of *Atm*^{-/-} mice and human cells from A-T subjects (19, 23). Conversely, DNA deletion frequencies of *Atm*^{-/-} mice housed under SPF-N conditions were 43%, a level even higher than those observed in the Harvard colony (Figure 1a). *Atm*^{-/-} mice housed under SPF-N conditions also had an increased level ($P < 0.05$) of DNA deletions compared to their wildtype littermates (Figure 1a).

Focusing on the two UCLA *Atm*^{-/-} mouse colonies, we then compared lifespan and lymphoma latency. SPF-N mice exhibited significantly shorter lifespans than SPF-S mice (44 vs 51 weeks, Figure 1b). Since *Atm*^{-/-} mice primarily die from lymphomas (6), we examined the cause of mortality or morbidity. Although the incidence of lymphomas was similar between mice housed in SPF-N (74%) and SPF-S (76.5%) conditions, time of onset was significantly shorter in the SPF-N mice (Figure 1c). The median age at which SPF-N mice died of lymphoma was 25 weeks compared to 60 weeks for SPF-S mice. These results are consistent with the observed increase in SPF-N genetic instability (Figure 1a), likely the primary driver of accelerated carcinogenesis. The decreased lymphoma latency in SPF-N mice was also the major cause of the decreased lifespan since the lymphoma-free mice in the SPF-N and SPF-S conditions had similar lifespans (data not shown).

After observing the striking effects of housing on ATM-deficient traits, we examined the intestinal bacteria from fecal pellets of *Atm*^{+/-} mice in these two UCLA mouse colonies. High throughput sequence analysis of bacterial 16S rRNA genes revealed that these two colonies harbor distinct microbial communities (Figure 1d). An indicator analysis identified several species-level phylotypes whose populations were higher in CM versus RM mice,

suggesting a possible causative role in carcinogenesis. One correlate is a member of the Helicobacteriaceae, which are bacteria known to promote cancer (Figure S1).

***Atm*^{-/-} mice with restricted microbiota (RM) have an increased lifespan and decreased systemic genotoxicity and oxidative stress**

To further investigate the role of intestinal microbiota in lymphoma penetrance, we created *Atm*^{-/-} mice harboring restricted (RM) and conventional microbiota (CM), which were created by rederivation as described in Fujiwara *et al.* (17) and by antibiotic treatment (18) followed by orogastric gavage of CM feces, respectively. These models were chosen because CM and RM harbor distinct intestinal microbiota (17, 22, 24), and RM mice possess immunologic traits that can potentially influence lymphoma penetrance, including unusually high levels of cytolytic central memory CD8⁺ T cells that target neoplastic cells. In addition, since RM mice are physically isolated from other colonies, their immunologic traits have persisted for many years, providing a stable platform for our investigations.

Lifespan in the two mouse colonies was significantly different, with median survival of CM mice (31.1 weeks) being shorter than RM mice (45.6 weeks) (Figure 2a). Examination of systemic genotoxicity using different metrics showed that *Atm*^{-/-} mice with CM microbiota exhibited higher levels of DNA damage than those with RM microbiota. Similar differences were not found between SPF-N and SPF-S mice. Clastogenic DNA damage, determined by the presence of micronuclei in peripheral blood erythrocytes, was nearly 85% higher in CM versus RM mice (Figure 2b). DNA strand breaks, measured by the alkaline comet assay, were also higher in CM than RM mice (Figure 2c). Levels of DNA strand breaks were determined by the Olive tail moment (Figure 2c) and confirmed by analysis of percent tail DNA at the 80th percentile (data not shown).

Since oxidative stress can be produced by intestinal microbiota both directly and indirectly (25, 26), and oxidative stress is associated with both genotoxicity and carcinogenesis (27), we also assessed the antioxidant state of *Atm*^{-/-} mice harboring CM and RM microbiota. Glutathione (GSH) is a major cellular antioxidant linked to several diseases including cancer. A metric of oxidative stress can be expressed as the ratio of GSH to GSSG (the dimeric oxidized form) in peripheral blood. We found that *Atm*^{-/-} mice harboring CM microbiota had higher levels of oxidative stress (lower GSH/GSSG ratios) compared to RM mice (Figure 2d), suggesting it may also play a role in lymphoma penetrance in this model system.

The results presented in Figure 1 indicate that sterility may play a role in carcinogenesis in *Atm*^{-/-} mice; the results in Figure 2 suggest that the intestinal microbiota is a major contributor to the phenotypic differences observed within and among isogenic *Atm*^{-/-} mouse colonies (9).

Microbiota in CM and RM mice are distinct

A high throughput sequence analysis revealed broad taxonomic differences in bacterial rRNA gene composition from the intestinal mucosa of CM and RM mice. We performed this analysis because all prior investigations of CM and RM microbiota were relatively shallow in depth (17, 22, 24). A UniFrac analysis showed that the bacterial communities between CM and RM mice are distinct (Figures 3a and 3b). The majority of rRNA gene sequences were classified into the phyla Bacteroidetes/Chlorobi (purple, Figure 3c) and Firmicutes (light green, Figure 3c). However, the most consistent phyla-level difference between CM and RM was in the Proteobacteria (sage green with asterisks, Figure 3c), with the most statistically significant differences occurring in the *Atm*^{-/-} mice (Figure 3d). Although compositional differences were detected between mouse genotypes (*Atm*^{-/-}

versus *Atm*^{+/+}) and intestinal regions (small intestine versus colon), CM versus RM provided the most distinct groupings (Figures 3a and 3b). A more comprehensive analysis is provided in the supplement (Table S2).

An analysis of the rRNA gene sequences at a finer taxonomic level revealed several operational taxonomic units (OTUs) from the intestinal mucosa that were differentially abundant in CM and RM mice (Figure 4a). We consider differentially abundant OTUs worthy of further study because they might represent individual bacterial species participating in the enhancement or inhibition of systemic genotoxicity or other pertinent CM-RM metrics. The closest BLAST hit of one of the most abundant OTUs in many RM habitats was *Porphyromonas asaccharolytica* (98% identity to CP002689, 43% coverage) (light blue with black asterisks, Figure 4a). This phylotype was significantly more abundant in RM than CM *Atm*^{-/-} mice in both the small intestine and colon, and it comprised at least 50% of the total sequencing reads from these habitats (Figure 4b), warranting further investigation as a potential inhibitor of systemic genotoxicity. Similarly, another OTU exhibiting higher populations in RM habitats of *Atm*^{-/-} mice (Figure 4c; and maroon with white asterisks, Figure 4a) was identical to *Lactobacillus johnsonii* (100% identity to CP002464, 100% coverage). A phylotype that was more abundant in CM than RM mice, and therefore a candidate for causing the observed genotoxicity, included a member of the Helicobacteriaceae (Table S3). A more comprehensive OTU analysis is provided in the supplement (Table S3).

***Lactobacillus johnsonii* decreases systemic inflammation and genotoxicity in *Atm*^{-/-} mice**

To verify whether individual bacteria were contributing to the differential systemic genotoxicity detected in CM and RM mice, we performed oral inoculation experiments. The high throughput sequence analysis had identified numerous phylotypes exhibiting higher populations in RM than CM mice – a feature suggesting a possible beneficial role. We attempted to culture two of the most abundant of these (Figures 4b and 4c), and successfully isolated and grew *L. johnsonii* from RM mice in pure culture; the rRNA gene sequence of our *L. johnsonii* isolate is identical to the one identified by the high throughput sequence analysis (Figure 4c).

CM *Atm*^{-/-} mice were orally gavaged with 10⁹ CFU of *L. johnsonii* every other day for 4 weeks. Fecal qPCR targeting *L. johnsonii* demonstrated again the deficiency of this phylotype in CM *Atm*^{-/-} mice, and also that periodic administration resulted in successful establishment and maintenance of high enteric levels (Figure 5a). After this 4-week period, *L. johnsonii* but not the vehicle control (PBS) resulted in reduced micronucleus levels (Figure 5b); however levels of DNA strand breaks were not significantly changed as measured by the comet assay or γ H2AX focus formation (data not shown). This was a time-dependent effect, as no difference was observed after only 1 or 2 weeks of treatment (Figure S2a).

Since systemic genotoxicity is induced by innate inflammatory mediators (20, 28), we analyzed the effect of *L. johnsonii* administration on basal systemic inflammatory parameters in these mice. In the liver, *L. johnsonii* significantly reduced the abundance of both hepatic NK and T cells (Figures 5c, d). A comparable reduction of these leucocyte subsets was also observed in the splenic and blood compartments (Figures S2b, c). With respect to molecular mediators, *L. johnsonii* treatment significantly reduced levels of the pro-inflammatory cytokines IL-1 β and IFN- β , and elevated the levels of the anti-inflammatory cytokines TGF β and IL-10 (Figure 5e). Similar changes were also observed in the blood compartment (Figure S2d).

These findings indicate that short-term administration of the single RM-associated bacterium, *L. johnsonii*, recapitulated the reduction of systemic micronucleus formation observed in RM mice, a host effect associated with reduced systemic inflammatory activity.

Discussion

Our results are the first to show a relationship between intestinal microbiota and lymphoma onset. In addition, these investigations generated a detailed catalog of bacterial phylotypes that are differentially abundant between CM and RM mice, thereby providing candidates that may influence a wide range of traits from systemic genotoxicity (this study), oxidative stress (this study), colitis resistance (29), pathogen clearance (30) and selective reduction of marginal zone (MZ) B cells (31), plasmacytoid dendritic cells (pDC) (17), and invariant natural killer (iNK) T cells (24). Moreover, we isolated one highly enriched species, *L. johnsonii*, from RM mice and subsequently demonstrated its ability to decrease systemic inflammation and micronucleus formation in *Atm*^{-/-} mice via oral inoculation experiments. Below, we propose several mechanistic hypotheses of how *L. johnsonii* might influence these important host traits.

First, *L. johnsonii* may reduce systemic genotoxicity by inhibition of basal intestinal inflammatory activity and its systemic sequelae. Our recent work revealed that intestinal inflammation-associated genotoxicity occurs not only locally, but also systemically. Using either a model chemical inflammatory agent, dextran sodium sulfate (DSS), or immune-mediated genetic models to induce local intestinal inflammation, we found that systemic genotoxicity was elevated in peripheral lymphocytes, an effect amplified in *Atm*^{-/-} vs. wildtype mice (20). Moreover, such lymphocyte genotoxicity is particularly abundant in the B lymphocyte subset, the progenitor cell type for B cell lymphoma (the predominant cancer in A-T patients) (32). Genetic and intervention studies have revealed that one factor is systemic dispersal of the intestinal cytokine TNF α , which permits genotoxicity in TNF α receptor-bearing lymphocytes remote from the intestinal compartment, in a process dependent on cell-autonomous, NF κ B and AP-1 induction of reactive oxygen and nitrogen species (RONS) (28).

The present study documents the reduction of several systemic measures of inflammatory activity after *L. johnsonii* restoration in CM *Atm*^{-/-} mice. There are several known anti-inflammatory mechanisms of *Lactobacillus* spp. that may account for this host response, including products that directly modulate the NF κ B inflammatory program of mucosal epithelial and hematopoietic cell types, or indirectly by ecologically changing the composition or functional activity of enteric microbial community (33, 34). Our study also documented that enteric *L. johnsonii* resulted in a reduction in the abundance of hepatic and migratory (blood, splenic) NK and T cells; a reduction also concordant with their reduced levels in CM colonies (17, 31). The control of NK and T cells in these compartments is complex, integrating a diversity of chemoattractant and trophic cytokines. *Lactobacillus* spp. may either augment or reduce the production of these key cytokines, by parenchymal epithelial and hematopoietic cells, depending on differences in host compartment and *Lactobacillus* spp. (34, 35). The present study suggests that enteric (dietary) administration of *L. johnsonii* may represent a viable strategy to reduce inflammation-induced genotoxicity.

The ability of *L. johnsonii* to reduce pathogen-associated inflammation has been demonstrated in several prior *in vivo* investigations, including two involving *H. pylori*, a known cancer-promoting bacterium. In experiments examining *H. pylori* infection in C57BL/6 mice, oral administration of *L. johnsonii* over a 3-month period reduced the amounts of lymphocytic and neutrophilic infiltration of the lamina propria as well as proinflammatory chemokines (36). Similarly, *L. johnsonii* reduced both *H. pylori*

populations and gastritis in Mongolian gerbils (37). A single inoculation of 1-day-old chickens with *L. johnsonii* inhibited the colonization and persistence of *Clostridium perfringens*, a poultry pathogen that causes necrotic enteritis (38). Finally, prior inoculation of gerbils with *L. johnsonii* prevented a persistent infection by the protozoan parasite, *Giardia intestinalis* (39).

Second, analysis of the literature suggests that *L. johnsonii* reduces immune-mediated oxidative stress and systemic genotoxicity by decreasing NF- κ B activation. Linked to cancer and various inflammatory disorders, NF- κ B is involved in managing responses to a wide range of potentially deleterious stimuli. Consistent with an agent that down-regulates NF- κ B activation, *L. johnsonii* and other CM-RM associated microbiota may affect lymphoma penetrance by altering systemic genotoxicity caused by immune-mediated oxidative stress. Our results and others demonstrate that oral administration of *L. johnsonii* reduces both oxidative stress (40, 41) and systemic genotoxicity (this study). Mechanistically, there are precedents that microbial environments modulate cancer formation in part through oxidative stress mediated by inflammatory or carcinogenic bacterial metabolites on local epithelial cells (15, 42). For example, inflammation that accompanies *H. pylori* infection, *S. haematobium* infection, or human inflammatory bowel disease is associated with elevated risk of stomach, bladder, or colon cancer, respectively (42). In an experimental system examining vaginal infection of mice with the bacterium *Gardnerella vaginalis*, oral administration of *L. johnsonii* reduced levels of proinflammatory cytokines, oxidative stress (iNOS), and activation of NF- κ B (41). An important factor linking inflammation to neoplasia is genotoxicity from inflammation-associated oxidative products, created either in trans (due to local oxidative products of inflammatory cells), and cell-autonomously (due to endogenous intra-cellular oxidative products induced by receptors to TNF α and other cytokines) (43, 44). In disease models, enteric colonization of *H. hepaticus* elicits innate immune activation, which via TNF- α and iNOS induction yields host oxidative products required for neoplasia.

Finally, genomic analysis of *L. johnsonii* revealed several features that may contribute to a superior colonizing ability in the mucosa, and to its ability to outcompete pathogens and other proinflammatory organisms (45). Attachment to the host is often a key feature of mucosa-associated microbes. Putative cell-surface proteins in *L. johnsonii* have similarity to the mucin binding protein (MUB) from *Lactobacillus reuteri* (46). In addition, similarities to Fap1 and GspB from *Streptococcus* species suggest *L. johnsonii* encodes adhesive and fimbrial proteins, respectively (47, 48). *Lactobacillus johnsonii* also produces a putative cell-surface protein with similarity to an IgA protease, which could enable it to avoid a key host defense mechanism. In addition, *L. johnsonii* may inhibit potential microbial competitors by producing the bacteriocin Lactacin F and by increasing Paneth cell numbers, which are a host cell type that produces antimicrobial compounds (49-50).

In sum, these investigations build on prior work linking intestinal microbiota, via control of basal levels of inflammation and oxidative stress, to systemic genotoxicity and carcinogenesis. Given that intestinal microbiota is a potentially modifiable trait, these and related insights hold considerable promise for translational intervention of B cell lymphoma and other diseases driven by immune-mediated oxidative stress and its resulting systemic genotoxicity. The translational promise of this approach, exemplified by the present study, suggests that simple interventions such as sustaining enteric levels of *L. johnsonii*, may favorably shift microbial composition and function to reduce basal levels of genotoxicity in a manner that may reduce cancer risk in susceptible individuals, such as those bearing the A-T genotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank N. Carls, D. Cowan and L. Parfenova for technical assistance. This work was supported by NIH grants ES09519 (RHS), CA133928 (RR), AI078885 (JBorneman), DK46763 (JBraun), P30 CA016042 (Jonsson Comprehensive Cancer Center), the Crohn's and Colitis Foundation of America 3153 (JBraun), the Broad Center of Regenerative Medicine and Stem Cell Research (JBraun), the Austrian Federal Ministry of Science and Research (GEN-AU III InflammoBiota) (DB, AL), NASA grant NNX11AB44G (RHS, JBorneman), the Ruzic Research Foundation (JBraun), and dissertation fellowships from UC TSR&TP and UCLA Graduate Division (MLY). Drs. Braun, Borneman, and Schiestl are filing a patent application related to this study.

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Figure 1.

Genetic Instability, Lymphoma Latency, and Lifespan are Increased in Separate Isogenic Colonies of *Atm*^{-/-} Mice

(A) Genetic instability (DNA deletions) were measured by P^{un} reversion assays (n = 22, 83 and 62 for Harvard, SPF-S and SPF-N, respectively).

(B) Kaplan-Meier survival curve (n = 34 and 31 for SPF-S and SPF-N, respectively).

(C) Lymphoma latency was determined for the subset of mice developing lymphoma (n = 13 and 15 for SPF-S and SPF-N, respectively).

(D) An unweighted UniFrac analysis of bacteria in *Atm*^{+/-} mice in SPF-S (light spheres) and SPF-N (dark spheres) conditions. Spheres around points (individual mice) indicate the 95% confidence ranges estimated by bootstrap resampling of data sets at 1300 reads.

Figure 2.

Lifespan, Genotoxicity, and Oxidative Stress of *Atm*^{-/-} Mice Bearing CM and RM Microbiota

(A) Kaplan-Meier survival of CM and RM *Atm*^{-/-} mice (n = 38 and 31 for CM and RM mice, respectively).

(B) Peripheral blood erythrocyte micronuclei levels (n = 5 and 6 for CM and RM mice, respectively).

(C) Olive tail moments in blood leucocytes (n = 5 for both groups).

(D) Oxidative stress measured by GSH/GSSG ratios (n = 3 and 2 for CM and RM mice, respectively). All measurements were in mice aged 4-6 months.

Error bars indicate SE.

Figure 3. Intestinal Microbiota in CM and RM Mice are Distinct at the Community and Phyla Levels (A, B) A normalized weighted UniFrac analysis of bacteria in mice in CM and RM conditions. (C) Area plot of phyla in CM and RM mice by genotype and intestinal region. Colored rectangles at bottom designate phyla type. Asterisks in area plot designate Proteobacteria. See Supplemental Figure S3 for a complete Phyla Legend. (D) Distribution and abundance of Proteobacteria by genotype and intestinal region. For CM, $n = 3, 7, 3, 7$ for small intestine (SI)- $Atm^{+/+}$, SI- $Atm^{-/-}$, colon (CLN)- $Atm^{+/+}$, and CLN- $Atm^{-/-}$, respectively. For RM, $n = 3, 4, 3, 4$ for SI- $Atm^{+/+}$, SI- $Atm^{-/-}$, CLN- $Atm^{+/+}$, and CLN- $Atm^{-/-}$, respectively. Differences were assessed by Mann-Whitney U tests.

Figure 4.

Intestinal Microbiota in CM and RM Mice are Distinct at the OTU Level

(A) Area plot of the most abundant OTUs in CM and RM mice by genotype and intestinal region. Black and white asterisks designate *P. asaccharolytica* and *L. johnsonii*, respectively.

(B) Distribution and abundance of *Porphyromonas asaccharolytica* by genotype and intestinal region. Differences were assessed by Mann-Whitney U tests.

(C) Distribution and abundance of *Lactobacillus johnsonii* by genotype and intestinal region. Differences were assessed by Mann-Whitney U tests.

See Figure Legend 3 for mouse numbers per genotype and intestinal region.

Figure 5.
Effect of Oral Administration of *L. johnsonii* on Genotoxicity and Inflammation in CM *Atm*^{-/-} Mice
(A) Fecal levels of *L. johnsonii* were quantified by qPCR.
(B) Micronucleus quantification in RBCs.
(C, D) Levels of hepatic NK (C) and T cells (D) were quantitated by flow cytometry for CD335 and CD3 cells, respectively.
(E) Hepatic tissue levels of cytokines were measured by ELISA, and fold-change was calculated for *L. johnsonii* relative to vehicle (PBS) control groups.
Error bars indicate SE.
* = $P < 0.05$ by Student's *t* tests.