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Enterococcus faecalis Induces A neuploidy and Tetraploidy in Colonic Epithelial Cells through a Bystander Effect

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Abstract

Intestinal commensals are potential important contributors to the etiology of sporadic colorectal cancer but mechanisms by which bacteria can initiate tumors remain uncertain. Herein we describe mechanisms that link *Enterococcus faecalis*, a bacterium known to produce extracellular superoxide, to the acute induction of chromosomal instability. Immortalized human and non-transformed murine colonic epithelial cells, along with a mouse colonic ligation model, were used to assess the effect of *E. faecalis* on genomic DNA stability and damage. We found that this human intestinal commensal generated aneuploidy, tetraploidy, and γH2AX foci in HCT116, RKO, and YAMC cells. In addition, direct exposure of *E. faecalis* to these cells induced a G2 cell cycle arrest. Similar observations were noted by exposuring cells to *E. faecalis*-infected macrophages in a dual-chamber co-culture system for detecting bystander effects. Manganese superoxide dismutase, catalase, and tocopherols attenuated, caffeine and inhibitor of glutathione synthase exacerbated, aneugenic and cyclomodulatory effects and linked the redox-active phenotype of this intestinal commensal to potentially transforming events. These findings provide novel insights into mechanisms by which *E. faecalis* and intestinal commensals can contribute to cellular transformation and tumorigenesis.

Keywords

aneuploidy; chromosomal instability; *Enterococcus faecalis*; DNA damage; G2 phase cell cycle arrest

INTRODUCTION

Intestinal commensals are potential important contributors to the development of sporadic human colorectal cancer (CRC), but the identification of relevant microorganisms has been impeded by the complex ecology of the large intestine (1,2). Several bacteria are definitively linked to gastrointestinal tumorigenesis; for example, *Helicobacter pylori* is considered a class I carcinogen for gastric cancers (3). However, in contrast to the stomach where the microbiota only consists of *Helicobacter* spp., the human colon contains >400 species with $>10^{11}$ colony forming units (cfu) per gram of feces (4). At least 75% of these microorganisms cannot be

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cultivated using current techniques and therefore remain largely uncharacterized. These difficulties have made the epidemiological approach to identifying CRC-associated microorganisms problematic. An alternate approach involves focusing on commensals that can confer long-term exposure to the host and are potentially mutagenic.

Enterococcus faecalis is a minority constituent of the intestinal microbiota that uniquely produces reactive oxygen species (ROS) including extracellular superoxide (5). This phenotype is rare among bacteria and known to damage colonic epithelial DNA (6). *E. faecalis* also causes inflammation and CRC in interleukin-10 knockout mice (7,8), and promotes chromosomal instability (CIN) in mammalian cells (9). CIN is the most common form of genomic instability in solid tumors. It consists of gains and losses of chromosome segments, or whole chromosomes, along with inversions, translocations, and complex rearrangements in the genome sequence and structure (10). The dramatic and unstable changes in genomic content for cells with CIN are typified by aneuploidy. Recent mathematical modeling and experimental evidence suggest that CIN is an early event in tumorigenesis (11, 12). The origin of CIN, however, remains uncertain. We recently showed that *E. faecalis* promotes CIN through free radical mechanisms initiated by superoxide (9). This effect was linked to expression of cyclooxygenase (COX)-2 in macrophages and production of clastogens (or chromosome breaking factors) that diffuse into neighboring cells to damage DNA.

The production of clastogens by *E. faecalis*-infected macrophages is analogous to the induction of CIN through the radiation-induced bystander effect (13). This effect is generated when cells are activated by radiation to produce diffusible mediators that damage DNA in neighboring unirradiated cells. The bystander effect has been shown to occur in vivo in congenic sex-mismatch bone marrow transplantation experiments (14). Although causative mediators are not characterized, the bystander effect is associated with COX-2 and DNA damaging products from this inflammatory pathway may be important (9,15,16).

A macrophage-induced bystander effect is consistent with the potential contribution of innate immune cells to tumorigenesis through prostaglandins, cytokines, and chemokines that have transforming, proliferative, and metastatic consequences (17). In this study we found that *E. faecalis* induced aneuploidy, tetraploidy, and DNA damage in colonic epithelial cells. Superoxide production was associated with DNA double strand breaks (DSBs), G2 arrest, and changes in chromosome number. These findings occurred through a bystander effect and provide a mechanism by which redox-active intestinal commensals may contribute to cellular transformation and tumorigenesis.

MATERIALS AND METHODS

Cell Lines and Bacterial Strains

HCT116 and RKO cells are near-diploid human colonic epithelial cell lines (43-45 and 46-49 chromosomes per cell, respectively) with defective mismatch repair (American Type Culture Collection, Manassas, VA) (18). These cell lines were maintained in McCoy's 5A (Invitrogen, Carlsbad, CA) containing 10% FBS, penicillin G, and streptomycin and have stable karyotypes (18,19). YAMC cells are a non-transformed line from the Ludwig Institute for Cancer Research and were grown in 5% CO_2 at 33°C using RPMI-1640 (Invitrogen) supplemented with 5% FBS, recombinant murine interferon-γ (Peprotech, Rocky Hill, NJ), and ITS Premix (20). RAW264.7 cells (ATCC) are murine macrophages and were maintained as previously described (9).

E. faecalis OG1RF is a human isolate that produces extracellular superoxide (21). Among healthy adults, *E. faecalis* varies in concentration in feces from $<10^5$ to 10^8 cfu per gram (22). This strain was grown in brain-heart infusion (Difco) at 37°C and washed in PBS with

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the multiplicity of infection (MOI) defined as bacterial cfu per cultured cell. Superoxide production was verified using ferricytochrome *c* reduction (5). MnSOD was purchased from Roche (Indianapolis, IN) and additional reagents from Sigma unless otherwise specified. Tocopherols (Tama Chemicals, Kawasaki, Japan) and γ-carboxyethyl hydroxychroman (Encore Pharmaceuticals, Inc, Riverside, CA) were provided by Kenneth Hensley.

For cells directly exposed to *E. faecalis*, a 2 hr treatment was carried out in medium without serum or antibiotics, and restored to complete medium after treatment. The pH of the medium during experiments was maintained in a physiological range during co-culture with *E. faecalis*. Antioxidants and inhibitors were added with *E. faecalis* in experiments as specified. Experiments with the dual-chamber co-culture system were carried out as previously described (9).

Cytogenetics

Anaphase bridging was analyzed using cells grown on CultureSlides (BD Biosciences, Bedford, MA). Following 24 hr incubation, cells were fixed with 2% paraformaldehyde and stained with propidium iodide. Anaphases were scored by one of us (X.W.) using fluorescent microscopy according to previously described criteria (23).

FACS Analysis for A neuploidy and Tetraploidy

Ploidy was determined by FACS analysis as previously described (24). In brief, cells were treated with 1×10^9 cfu ml^{-1} *E. faecalis* for 2 hrs. After 48 hrs, cells were arrested in mitosis with colcemid, fixed (70% ethanol), and stained with Phospho-Histone H3 (Ser^{10}) mouse monoclonal antibody (1:400 dilution, Cell Signaling Technology, Danvers, MA). Phosphorylation of histone H3 is a marker of chromosome condensation and mitosis. Cells were secondarily stained with the Alexa Fluor® 488 F(ab')2 fragment of goat anti-mouse IgG (H+L) (1:1000 dilution, Invitrogen) and propidium iodine. Cell sorting was performed on FACSCalibur (BD Bioscience) using CellQuest™ software.

To investigate the induction of aneuploidy, *E. faecalis*-exposed HCT116 cells were double-stained with Hoechst 33342 and propidium iodide. Cells with DNA content >5N were sorted as single cells into wells onto 96 well plates (inFlux Cell Sorter, Cytopeia, Seattle, WA) and incubated for 2 weeks. Colonies were enumerated and compared to untreated controls.

Cell Cycle Assay

Cells were synchronized in G1 using 2 mM thymidine, followed by thymidine-free medium, and then thymidine-supplemented medium (25). Cells were treated with 1×10^9 cfu ml^{-1} *E. faecalis* for 2 hrs (MOI = 1,000) and fixed. For the dual-chamber system, uninfected and *E. faecalis*-infected macrophages were placed in the upper compartment (9). *E. faecalis* is readily phagocytosized by macrophages and survives as an intracellular pathogen (26). Synchronized cells in the lower compartment were used as targets, fixed, stained with propidium iodine, and sorted by FACS. Data were analyzed using ModFit LT (Verity Software House, Topsham, ME).

Western Blotting

Protein extraction and blotting were carried out using ECL (GE Healthcare). Phosphorylated-ATM (Ser^{1981}), p-ATR (Ser^{428}), p-Chk1 (Ser^{345}), p-Chk2 ($Ser^{33/35}$) and p-Cdk1 (Tyr^{15}) monoclonal antibodies and polyclonal anti-cyclin B1 antibody were purchased from Cell Signaling Technology. Murine anti-β-actin loading control was purchased from BioVision (Mountain View, CA). Donkey anti-mouse IgG HRP conjugate (Santa Cruz Biotechnology) and goat anti-rabbit IgG HRP conjugate (Zymed) were used as secondary antibodies.

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In vivo DSBs Caused by *E. faecalis*

To assess the DNA damaging effect of *E. faecalis* in vivo we developed a colonic ligation model (27). Colons of wild-type BALB/c mice were surgically filled 1.0 ml of PBS as a control ($n = 3$) or *E. faecalis* at 1×10^8 cfu ml^{-1} in PBS ($n = 3$) and 5 mM D-glucose added to initiate superoxide production (5). Colon contents were retained using a rectal suture and ligature at the proximal colon. Care was taken to preserve blood flow with no ischemia observed in the colons of 24 mice previously studied using this protocol (27). At 6 hrs post-inoculation colons were removed for analysis.

Wild-type C57BL/6 mice were subjected to 13 Gy whole body radiation in a ^{137}Cs irradiator. Animals were euthanized 4.5 hrs following irradiation and colons removed for analysis. Protocols were approved by animal study committees of the University of Oklahoma Health Sciences Center and Oklahoma City VA Medical Center.

Immunofluorescence and immunohistochemistry

HCT116 and RKO cells treated with *E. faecalis* were fixed with 2% paraformaldehyde and stained using Phospho-Histone H2AX (Ser^{139}) rabbit monoclonal antibody conjugated to Alexa Fluor® 488 (Cell Signaling). DNA was counterstained with 4′-6-diamidino-2-phenylindole. γH2AX-positive cells were identified by laser scanning confocal microscopy (Leica Microsystems, Bannockburn, IL) or enumerated by FACSCalibur and analyzed using Summit software (v4.3, DAKO, Carpinteria, CA).

Epitope retrieval of paraffin-embedded colon sections was done using a pressurized decloaking chamber (Biocare Medical, LLC). Sections were blocked using Avidin/Biotin blocking kit (Vector Lab) and peroxidase activity quenched using 3% H_2O_2. After washing, slides were incubated in horse serum and bovine albumin and exposed to rabbit anti-phospho-H2AX antibody (Cell Signaling). Slides were incubated in biotinylated donkey anti-rabbit secondary antibody (Jackson Immuno Research Lab) and SA-HRP (Dako) and chromogenic development performed using DAB substrate (brown) with hematoxylin as the counterstain (Biocare Medical). Slides were scored for γH2AX in a blinded fashion by one of us (S.L.) counting only nuclear staining as positive.

Statistical Analysis

Data were expressed as means with standard deviations. Experimental and control groups in ploidy assays were compared by chi-square and the number of positive nuclei in colon biopsies by the student's t test. *P*-values < 0.05 were considered statistically significant.

RESULTS

E. faecalis Promotes Anaphase Bridging, Aneuploidy, and Tetraploidy

To assess mechanisms by which *E. faecalis* damages DNA and causes CIN, we initially evaluated the diploid and chromosomally stable HCT116 cell line for anaphase bridging after exposure to *E. faecalis*. Anaphase bridges appear as extended chromosomes between spindle poles during the separation of sister chromatids (23,28). HCT116 cells showed numerous abnormal mitotic patterns after exposure to *E. faecalis* including lagging chromosomes, anaphase bridges, and tripolar anaphases (Fig. 1*A*). We found a significant increase in the frequency of anaphase bridging following *E. faecalis* exposure [86 (57%) of 151 anaphases] compared to untreated control cells [35 (18%) of 194 anaphases; $P < 0.001$]. The frequency of anaphase bridging was significantly reduced when MnSOD was added [48 (30%) of 158; $P < 0.001$], suggesting that superoxide promoted abnormal mitoses in these cells.

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Since anaphase bridging can lead to aneuploidy (23), we next determined whether exposure to *E. faecalis* resulted in aneuploidy. Mitotic HCT116 cells were sorted using a monoclonal antibody to phosphorylated histone H3 and DNA content determined by FACS. Approximately 10% of untreated mitotic HCT116 cells were non-diploid with a DNA content greater or less than 4N (Fig. 1*B*, *left*). Following a 2 hr exposure to *E. faecalis*, the average frequency of non-diploid cells was significantly increased at 24, 48, 72, and 96 hrs post-exposure (Fig. 1*B*, *middle*, and Fig. 1*C*). This correlated with significant increases in both aneuploid and tetraploid (*i.e.*, 8N DNA content for cells in G2 or mitosis) cells (8.3% compared to 0.6% for untreated control; $P < 0.001$). Similarly, the percent of cells in G2 (*i.e.*, non-mitotic) with 8N DNA content increased following *E. faecalis* treatment compared to untreated controls (7.8% vs. 0.0%; $P < 0.001$). MnSOD was associated with a reduction in the number of mitotic aneuploid cells at 24, 48, and 72 hrs (Fig. 1*B*, *right*, and Fig. 1*C*). Reductions in mitotic cells, and cells in G2 with 8N DNA content, were also observed (8.2% to 3.5% and 7.8% to 2.0%, respectively, at 48 hrs). We next assessed whether tocopherols, scavengers of lipid radicals, protected against aneuploidy and tetraploidy. Addition of α- or γ-tocopherol significantly decreased the percentage of aneuploid cells compared to treatment with *E. faecalis* alone (Fig. 1*D*). Similar reductions were seen in the percentage of tetraploid cells. Notably, the tocopherol metabolite, γ-carboxyethyl hydroxychroman, a COX-2 inhibitor but poor scavenger (29), also decreased the frequency of aneuploidy (Fig. 1*D*).

To extend these findings, we repeated experiments using RKO cells. The percentage of aneuploid and tetraploid cells at baseline was similar to that for HCT116 cells and increased significantly following exposure to *E. faecalis* (Supplementary Fig. S1). In addition, the percentage of non-diploid cells decreased when MnSOD was added, suggesting that superoxide from *E. faecalis* promoted changes in chromosome number for both cell lines.

Errors in mitotic segregation, *e.g.*, due to anaphase bridging or mitotic failure, can result in aneuploidy and tetraploidy (10,30). To determine whether *E. faecalis* generated long-term changes in chromosome number, we exposed HCT116 cells to *E. faecalis* and selected highly aneuploid or tetraploid cells by FACS (*i.e.*, DNA content >5N). Single cells were sorted and after 2 weeks only 20 (6.9%) of 288 cells grew compared to 51 (17.7%) of 288 untreated cells ($P < 0.001$). Growth of untreated non-diploid cells was similar to untreated diploid cells with 60 (20.8%) of 288 cells viable ($P = 0.40$). *E. faecalis*-exposed clones were then somatically selected over 20 generations for heritable changes in DNA content. Reexamination by FACS detected only one tetraploid clone (Supplementary Fig. S2, *right*), with the remainder being diploid (Supplementary Fig. S2, *middle*). This implied that the acute induction of aneuploidy and tetraploidy by *E. faecalis* was unstable (or lethal) with few clones able to propogate only by reverting back to diploidy or, on occasion, maintaining tetraploidy.

E. faecalis Activates DNA Damage Responses and G2 Arrest

We noted that HCT116 and RKO cells developed megalocytosis following exposure to *E. faecalis* (data not shown). This effect was abrogated by catalase, but not MnSOD, and appeared similar to cytopathic effects produced by bacterial cyclomodulins known to interfere with the eukaryotic cell cycle. Cyclomodulins such as the cytolethal distending toxin delay entry into mitosis by damaging host cell DNA (25). We therefore analyzed by FACS HCT116 and RKO cells following exposure to *E. faecalis* and found that both developed arrest at the G2/M transition (Fig. 2*A*).

We hypothesized that *E. faecalis*-induced G2/M arrest was due to DNA damage from ROS and therefore investigated phosphorylations involved in checkpoint activation. ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) are kinases that initiate DNA damage response signal transduction cascades (31). Following DNA damage, ATM and/or ATR are activated by autophosphorylation and, in turn, phosphorylate Chk1 and/or Chk2

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(32-34). Chk1/Chk2 further phosphorylate Cdc25C leading to sequestration in the cytoplasm and failure to activate Cdk1 (Cyclin B dependent kinase 1), a key regulator of mitotic progression. Phosphorylation of Cdk1 at Thr^{161} and dephosphorylation at Tyr^{15}/Thr^{14} activates Cdk1 leading to progression from G2 to M (35). Western blots for p-ATM/p-Chk2 and p-ATR/p-Chk1 showed activation in HCT116 and RKO cells following exposure to *E. faecalis* (Fig. 2*B* and Fig. 2*C*). However, unlike HCT116 cells, RKO cells expressed p-ATR at baseline, suggesting constitutive activation as has been reported for many colon cancers (36). Despite the G2/M arrest following exposure to *E. faecalis* (Fig. 2*A*), phosphorylation of Cdk1 at Tyr^{15} failed to increase (Fig. 2*B* and Fig. 2*C*), although doxorubicin, the positive control, produced this effect. Cyclin B1 levels, in contrast, were markedly increased in treated cells compared to controls.

To investigate whether the spindle-assembly checkpoint contributed to *E. faecalis*-induced cell cycle arrest, we determined the proportion of cells containing phosphorylated histone H3 as a marker of chromosome condensation and mitosis. There was, however, no change in the proportion of cells with phosphorylated histone H3 following exposure to *E. faecalis* (Supplementary Fig. S3*A* and *B*). Nocodazole-treated cells, in contrast, showed a 5-fold increase by 24 hrs. This provided evidence that the spindle-assembly checkpoint was not activated by *E. faecalis*. Finally, caffeine, an ATM/ATR inhibitor (37), significantly increased the percentage of aneuploid and tetraploid cells following exposure to *E. faecalis* (Fig. 2*D*), and partially rescued cells from *E. faecalis*-induced G2 arrest (Supplementary Fig. S3*C*). In sum, these findings indicated that *E. faecalis*-induced G2 arrest did not occur through the canonical Cdk1 pathway and the elucidation of underlying mechanisms are under investigation.

E. faecalis Forms γH2AX Foci

Cellular responses to DNA damage include DNA repair cascades and, for severe injury, apoptosis. In mammalian cells γH2AX is rapidly formed by ATM at sites of DSBs. We identified γH2AX foci in the nuclei of cells following treatment with 1 μM doxorubicin, an inducer of DSBs, and following exposure to *E. faecalis* (Fig. 3*A* and Supplementary Fig. S4). The proportion of γH2AX positive cells was bimodal with peaks appearing immediately after exposure to *E. faecalis* and 48 hrs later (Fig. 3*B*). The proportion of γH2AX-positive cells was also noted to increase with greater MOIs (Fig. 3*C*). Finally, caffeine decreased the percentage (± SD) of cells positive for γH2AX (*E. faecalis* vs. *E. faecalis* with 2.5 mM caffeine: 43.4 ± 0.5 vs. 20.2 ± 1.8 for HCT116 cells, and 79.2 ± 0.2 vs. 67.4 ± 0.4 for RKO cells; $P < 0.01$ for both comparisons). These observations show that *E. faecalis* generates γH2AX foci and inhibition of ATM/ATR protects against *E. faecalis*-induced aneugenesis.

ROS Promote γH2AX Foci

The temporal pattern of γH2AX formation (Fig. 3*B*) suggested rapid DNA damage (38). To confirm ROS generated γH2AX foci and, presumably, DSBs, MnSOD and catalase were added to cells exposed to *E. faecalis*. Compared to *E. faecalis* alone, MnSOD partially protected against γH2AX formation in HCT116 cells at 0 hrs, but not 24 hrs (Fig. 3*D*, *upper panels*), and failed to protect RKO cells at any time point (Fig. 3*D*, *lower panels*). Catalase, however, prevented the formation of γH2AX foci, suggesting that the spontaneous dismutation of superoxide to H_2O_2 generated most of the DNA damage in these cells.

E. faecalis Induces DNA Damage and G2 Arrest in Non-transformed Cells

To determine whether induction of aneuploidy, tetraploidy, and G2 arrest by *E. faecalis* in HCT116 and RKO cells extended to non-transformed cells, we exposed YAMC cells to *E. faecalis*. A similar set of responses was found with increased aneuploidy and tetraploidy, γH2AX formation, decreased γH2AX foci following treatment with MnSOD and catalase, and G2 arrest (Supplementary Fig. S5). Caffeine decreased γH2AX foci (data not shown) and

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partially rescued YAMC cells from *E. faecalis*-induced G2 arrest (Supplementary Fig. S5). These findings confirmed the DNA damaging and cyclomodulatory effects of *E. faecalis* were not unique to transformed cells.

E. faecalis-infected Macrophages Induce Aneuploidy through a Bystander Effect

Colonic mucus limits direct contact between intestinal commensals and epithelial cells. Thus, luminal bacteria would likely need to exert promutagenic effects through indirect mechanisms. For *E. faecalis* this could occur via translocation of the intact colonic epithelium (39). We have shown in mice that this process can lead to acute changes in colonic mucosal gene expression and NF-κB activation in tissue macrophages (27). This finding is consistent with other work by us showing activated macrophages produce clastogens that damage epithelial cells via a mechanism analogous to the radiation-induced bystander effect (9,13). To investigate this hypothesis we exposed HCT116 cells to murine macrophages infected with *E. faecalis* in a dual-chamber tissue culture system (9). We found the percentage of aneuploid and tetraploid cells increased significantly at an MOI of 1,000 compared to uninfected controls and at lower MOIs (Fig. 4*A*).

Eukaryotic cells have numerous defenses against oxidative stress and to maintain genomic integrity. Glutathione (GSH) is a cofactor for enzymes that protect cells against free radical toxicity and xenobiotics. To determine whether GSH-dependent antioxidant defenses protected against the bystander effect, we depleted HCT116 cells of intracellular GSH using buthionine-sulfoximine (BSO), a specific inhibitor of GSH synthesis. Depleted cells were exposed to *E. faecalis*-infected macrophages in the dual-chamber culture system. As the MOI increased from 1 to 100 the proportion of aneuploid and tetraploid cells increased compared to uninfected macrophages (Fig. 4*B*). Cells treated with BSO alone showed no increase in the proportion of aneuploid and tetraploid cells. A slight increase was seen, however, in GSH depleted cells co-cultured with uninfected macrophages. This may represent basal production of clastogens by these cells (Huycke, unpublished data). Thus, GSH-dependent cellular defenses protected against clastogen-mediated aneugenesis and support the notion that *E. faecalis*-infected macrophages induce CIN through a bystander effect.

Infected M acrophages Cause DSBs through a Bystander Effect

To assess the response of colonic epithelial cells to *E. faecalis*-infected macrophages, we investigated DNA damage repair pathways using the dual-chamber co-culture system. Cells were synchronized in G1 by double thymidine block and exposed to *E. faecalis*-infected macrophages at an MOI of 1,000. HCT116 cells developed a marked G2/M arrest (Fig. 5*A, left*). The percentage of cells in G2/M was significantly increased at 48 hrs (8.0 ± 0.1) and 72 hrs (9.6 ± 0.5) compared to cells exposed to uninfected macrophages. In comparison, the percentage of RKO cells in G2/M did not change significantly (< 5%; Fig. 5*A, right*).

Activation of the G2 checkpoint by DSBs can occur through protein kinase damage response pathways. p-ATM/p-Chk2 and p-ATR/p-Chk1 signaling pathways were activated in both cell lines exposed to uninfected macrophages (Fig. 5*B* and Supplementary Fig. S6). Activation was moderately stronger, however, following exposure to *E. faecalis*-infected macrophages. As previously shown for cells directly exposed to *E. faecalis* (Fig. 2*B* and Fig. 2*C*), phosphorylation of Cdk1 at Tyr^{15} was evident at baseline and did not increase following exposure to *E. faecalis*-infected macrophages. A G2/M arrest, however, was only noted for HCT116 cells (Fig. 5*A*), suggesting differences in sensitivity to DNA damage or cell cycle checkpoints between these cell types.

We next identified γH2AX in cells exposed to supernatants from *E. faecalis*-infected macrophages (Fig. 5*C*, *lower panel*, and Supplementary Fig. S7). By laser scanning confocal

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microscopy γH2AX foci appeared more frequently in cells exposed to supernatants from infected macrophages than uninfected macrophages. Similarly, the proportion of γH2AX-positive cells at 5 hrs (data not shown) and 48 hrs (Fig. 5*D*) following exposure to infected macrophages was significantly greater than for cells exposed to uninfected macrophages. The proportion of γH2AX-positive RKO cells (Fig. 5*D*, *right*) was less than for HCT116 cells (Fig. 5*D*, *left*), suggesting that insufficient DNA damage as a potential explanation for the lack of arrest at G2. These showed that the bystander effect can activate ATM and ATR, generate γH2AX foci and, for HCT116 cells, induce a G2/M arrest.

E. faecalis Induces γH2AX Foci In Vivo

To determine whether *E. faecalis* could generate γH2AX foci in vivo, we developed an intestinal ligation model that exposed the colonic mucosa to an inoculum of enterococci. In this system wild-type mice were exposed to *E. faecalis* for 6 hrs, PBS as sham, or irradiated with 13 Gy. There was no histological evidence for inflammation or mucosal abnormality in control or *E. faecalis*-exposed mice. However, immunohistochemistry showed marked positivity for γH2AX in colonic epithelial and stromal cell nuclei for mice treated with *E. faecalis* or irradiated (Figs. 6*A*, 6*C*, and 6*D*). Comparison of *E. faecalis*-exposed colons to shams showed significant increases in γH2AX in the nuclei of epithelial and stromal cells (Figs. 6*B* to 6*D*). The average (± SD) number of positive nuclei per cross-section for surface epithelial, crypt epithelial, and stromal cells for *E. faecalis*-exposed compared to sham mice was 2.3 ± 1.7, 12.0 ± 7.2 and 3.1 ± 2.2 vs. 0.3 ± 0.5, 0.7 ± 1.2, and 0.0 ± 0.0, respectively ($P < 0.001$ for all comparisons). These results demonstrate that *E. faecalis* can generate rapid induction of γH2AX, and presumably DSBs, in colonic epithelial and stromal cells—findings not dissimilar to radiation-induced intestinal injury.

DISCUSSION

In this study we found that the human intestinal commensal *E. faecalis* induced γH2AX foci in human colonic epithelial cells, activated DNA damage pathways, produced G2 arrest, and promoted missegregation of chromosomes leading to aneuploidy and tetraploidy. The production of ROS by *E. faecalis* was associated with anaphase bridging, lagging chromosomes, and multipolar mitoses. MnSOD, catalase, and tocopherols attenuated aneugenic and cyclomodulatory effects, linking the redox-active phenotype of this commensal to these potentially transforming events. *E. faecalis* infection of macrophages produced a bystander effect that resulted in γH2AX deposition, cell cycle arrest, and CIN. Finally, the colonic mucosa of mice exposed to *E. faecalis* showed increased γH2AX formation similar to that produced by irradiation. γH2AX formation is a marker of DSBs and these findings support our previous work that *E. faecalis* can generate DNA damage in vivo (6). In general, DSBs are difficult to repair and can generate dicentric chromosomes through non-homologous end-joining repair (28). Dicentric chromosomes can attach to both spindle poles and lead to anaphase bridging. If bridges fragment, chromosomes can enter breakage-fusion-bridge cycles that produce rearrangements and aneuploidy (28). These are hallmark features of CIN.

We selected *E. faecalis*-exposed colonic epithelial cells with >5N DNA content to determine if aneuploid cells could persist and found that few survived clonal expansion. Of those that propogated, all reverted to near-diploid except for one clone that remained tetraploid. Indeed, the production of ROS by *E. faecalis* was associated with a significantly increased number of tetraploid cells. Polyploid cells with more than two sets of homologous chromosomes can, in general, arise following cellular stress through a failure of cytokinesis, by cell fusion, or from mitotic slippage (40). Tetraploid cells in particular are considered potential intermediates in the cellular progression from diploid to aneuploid. For example, supernumeray centrosomes in tetraploid cells can form multipolar mitoses and cause haphazard chromosome segregation.

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The tumorigenic potential of tetraploid cells was recently confirmed using a p53-deficient primary mouse mammary epithelial cell that, when exposed to cytochalasin, was rendered tetraploid and found to be malignant in nude mice (41).

E. faecalis-induced G2 arrest was partially ameliorated by MnSOD and indicated that superoxide can act as a cyclomodulin. Cyclomodulins are a family of secreted bacterial toxins that interfere with the eukaryotic cell cycle (42). One example, produced by extraintestinal pathogenic and commensal strains of *Escherichia coli*, is the cytolethal distending toxin that induces DSBs, cell distension, G2 checkpoint activation and, ultimately, cell death (25). Commensal bacteria with cyclomodulatory effects may initiate DSBs that lead to anaphase bridging, chromosomal fragmentation, and chromosomal rearrangements. G2 arrest should prevent cell cycle progression until DNA damage is repaired. However, damage below threshold values of 10-20 DSBs needed for the activation of mammalian checkpoints, would lead to propogation of chromosome aberrations to daughter cells (43). Evidence for this has been noted in sporadic CRC with mutations arising via low-level telomere-mediated anaphase bridging (23).

G2 arrest is typically induced by decreased dephosphorylation of Cdk1 at Tyr^{15}/The^{14} (44). However, by western blot, we found no evidence for Cdk1 inactivation in HCT116 or RKO cells exposed to *E. faecalis*. HCT116 and RKO cells are mismatch repair deficient, and this trait can negatively affect G2 checkpoint activation (45). Cell cycle arrest during G2 was confirmed by the persistence of cyclin B1 and lack of accumulation of cells in mitosis. However, classical activation of the G2 checkpoint through Cdk1 was not found and the G2 arrest that was induced by *E. faecalis* may have occurred through potential alternate pathways including p53/p21, GADD45, and 14-3-3σ among others (45,46). This is a subject of ongoing investigation.

We previously found that *E.faecalis* promoted CIN in colonic epithelial cells through a macrophage-induced bystander effect (9). As with radiation, superoxide is a trigger for this effect (47). COX-2 contributes to both the radiation- and macrophage-induced bystander effect (9,15,16), although mechanisms are unclear. The results reported here show that the macrophage-induced bystander effect can rapidly induce aneuploidy and tetraploidy in colonic epithelial cells. The bystander effect also generated γH2AX foci in target cells, a finding consistent with previous studies showing chromosomal DSBs due to clastogenesis (47). Depleting intracellular GSH with BSO amplified these effects suggesting that antioxidant or xenobiotic defenses can protect against clastogenesis and aneugenesis. Several potential mediators for the bystander effect include tumor necrosis factor-alpha and breakdown products of polyunsaturated fatty acids such as 4-hydroxy-2-nonenal (47,48), although no single agent has been definitively shown to be causative.

In conclusion, this study demonstrates that *E. faecalis* can induce γH2AX foci, G2 arrest, anaphase bridging, and aneuploidy and tetraploidy in human colonic epithelial cells through direct contact or following exposure to *E. faecalis*-infected macrophages. The latter is analogous to the radiation-induced bystander effect (13). Blocking DNA repair with caffeine, or depleting GSH-dependent defenses with BSO, significantly increased the frequency of aneuploidy and tetraploidy. Finally, *E. faecalis*-induced γH2AX formation in murine colons suggested that this commensal can generate DSBs in vivo. These findings provide insights into mechanisms by which commensal bacteria may contribute to the endogenous origin of CIN and cellular transformation.

Supplementary Material

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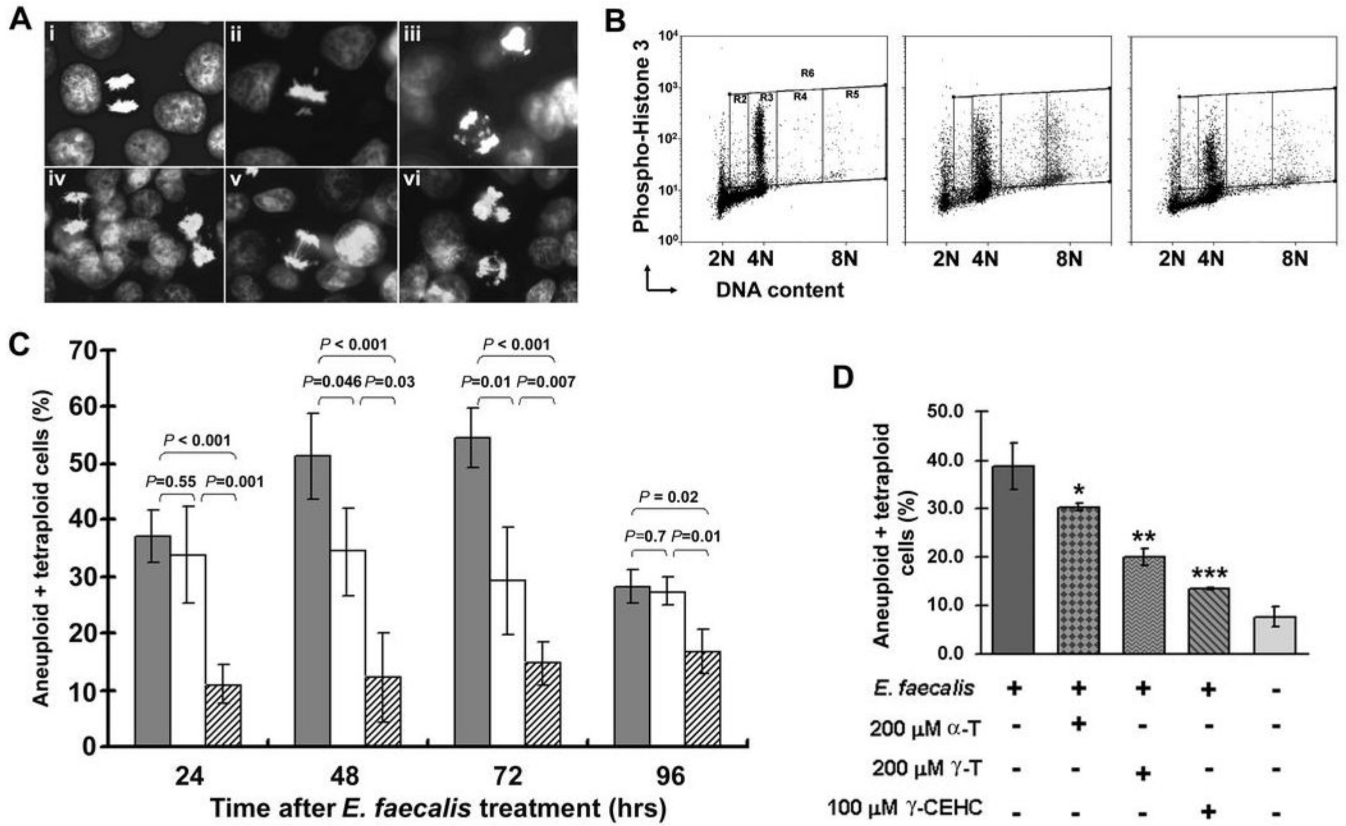

Figure 1. *E. faecalis* causes anaphase bridging, aneuploidy and tetraploidy

A, HCT116 cells after exposure to *E. faecalis*: normal anaphase (*i*); abnormal mitosis (*ii*); anaphases with lagging chromosomes (*iii-v*); and tripolar mitosis with bridging (*vi*). *B,* Histograms of FACS analysis for HCT116 cells: untreated cells (*left*); cells exposed to *E. faecalis* for 2 hrs (*middle*); and cells exposed to *E. faecalis* for 2 hrs with addition of MnSOD (*right*). Phosphorylated histone H3 antibody (Y-axis) with mitotic cells separated from interphase cells and propidium iodine for DNA content (X-axis). R2 gate contains cells with DNA between 2N and 4N (hypodiploid); R3 gate contains 4N DNA; R4 gate contains between 4N and 8N DNA; R5 gate contains >8N DNA. R2 + R4 + R5 represent the proportion of aneuploid and tetraploid cells. *C,* The proportion of aneuploid and tetraploid cells increase from 24 to 96 hrs after *E. faecalis* treatment (*gray bars*) compared to untreated control (*hashed bars*). MnSOD protects against aneuploidy and tetraploidy at 48 and 72 hrs (*white bars*). *D,* α-tocopherol, γ-tocopherol and γ-carboxyethyl hydroxychroman (γ-CEHC) decrease the frequency of aneuploidy and tetraploidy at 48 hrs compared to *E. faecalis* alone (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

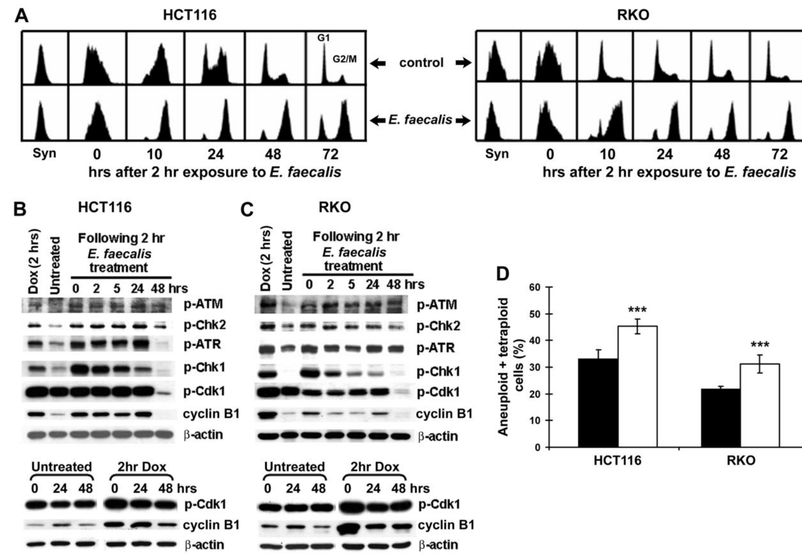

Figure 2. *E. faecalis* activates DNA damage responses

A, Cell cycle analysis shows *E. faecalis* induced a G2/M cell cycle arrest in HCT116 cells (*left*) and RKO cells (*right*). *Upper panel*: untreated control; *lower panel*: cells treated with 1×10^9 cfu ml^{-1} *E. faecalis* for 2 hrs. Syn: cells synchronized to G1 by double thymidine block. *B* and *C*, Western blots of p-ATM (Ser^{1981}), p-ATR (Ser^{428}), p-Chk1 (Ser^{345}), p-Chk2 ($Ser^{33/35}$), p-Cdk1 (Tyr^{15}) and cyclin B1. ATM/ATR are activated by transient infection with *E. faecalis* in (*B*) HCT116 cells and (*C*) RKO cells. Dephosphorylation of Cdk1 is evident at 48 hrs in both cell lines. Phosphorylation of Cdk1 at Tyr^{15} was seen in untreated cells for both cell lines (*lower panels*). *D* , Caffeine blocks ATM/ATR leading to increased frequency of aneuploidy and tetraploidy in *E. faecalis*-exposed HCT116 and RKO cells. Black bar: *E. faecalis*; white bar: *E. faecalis* with 2.5 mM caffeine (****P* < 0.001 compared to *E. faecalis* alone).

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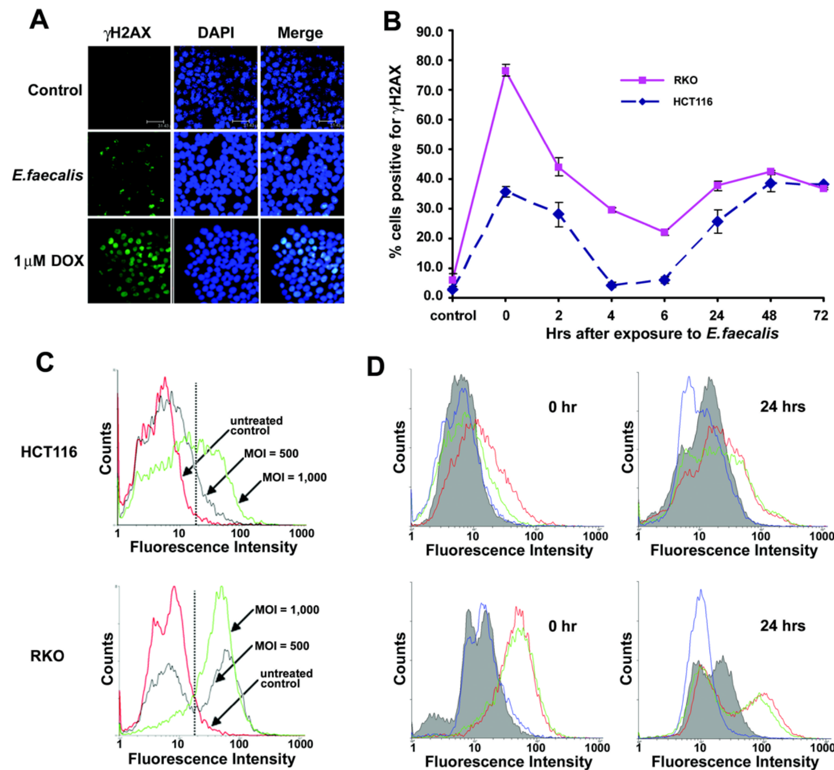

Figure 3. *E. faecalis* causes DSBs in colonic epithelial cells

A, Laser scanning confocal microscopy of HCT116 cells exposed to *E. faecalis* at an MOI of 1,000 shows DSBs as γH2AX foci (green nuclei). DNA was conterstained using DAPI (blue nuclei) with images merged (*right panels*). No γH2AX foci occur in untreated controls (*upper panels*). *B,* Analysis of γH2AX-positive cells by FACS shows DSBs following exposure to *E. faecalis* with a bimodal effect: blue trace, HCT116 cells; red trace, RKO cells. RKO cells are more sensitive to *E. faecalis*-induced DSBs at 0 and 6 hrs compared to HCT116 cells, but not beyond 24 hrs. *C, E. faecalis* causes DSBs in a dose-dependent manner for HCT116 cells (*upper panel*) and RKO cells (*lower panel*). Red trace: untreated control; gray trace: cells exposed to *E. faecalis* at a MOI of 500; green trace: MOI of 1,000. *D,* MnSOD is partially protective against DSBs in HCT116 cells at 0 hrs (*upper left*), but not 24 hrs (*upper right*) following exposure to *E. faecalis*. In contrast, MnSOD has no effect on the proportion of γH2AX positive RKO cells at 0 hrs (*lower left*) and 24 hrs (*lower right*). Catalase eliminates DSBs for HCT116 and RKO cells at all time points: gray trace: untreated control; red trace: MOI of 1,000; green trace: addition of MnSOD (1,200 units ml^{-1}); blue trace: addition of catalase (1,200 units ml^{-1}).

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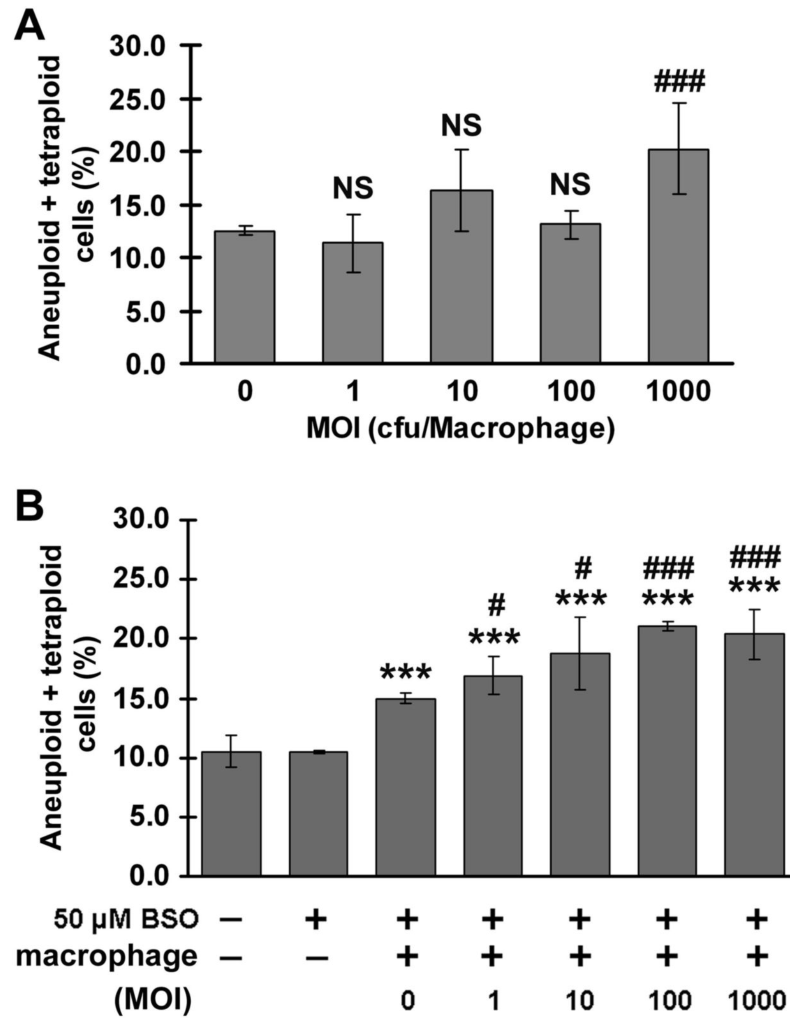

Figure 4. *E. faecalis*-infected macrophages induce aneuploidy and tetraploidy in colonic epithelial cells through a bystander effect

A, Macrophages infected with *E. faecalis* at an MOI of 1,000 increase the percent of aneuploid and tetraploid HCT116 cells ($^{###}P < 0.001$ compared to co-culture with uninfected macrophages; NS, not significant). *B,* Depletion of GSH in HCT116 cells by 50 μM BSO increases the percent of aneuploid and tetraploid cells when co-cultured with *E. faecalis*-infected macrophages at MOIs from 1 to 1,000 ($^{#}P < 0.05$ and $^{###}P < 0.001$ compared to exposure to uninfected macrophages (MOI = 0); $^{***}P < 0.001$, compared to without macrophages).

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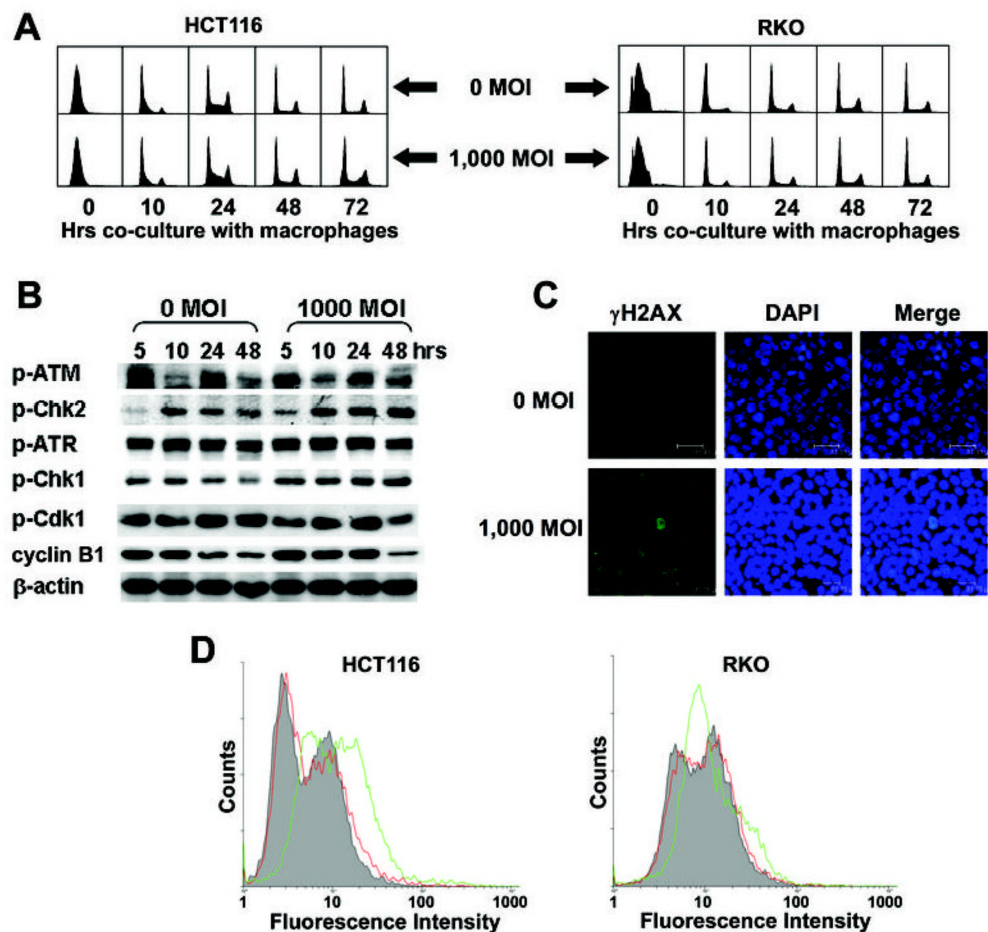

Figure 5. *E. faecalis*-infected macrophages induce DSBs in colonic epithelial cells through a bystander effect

A, Exposure of synchronized HCT116 cells to *E. faecalis*-infected macrophages at an MOI of 1,000 induce a G2/M arrest after 48 hrs compared to exposure to uninfected macrophages (*left*). Compared to co-culture with uninfected macrophages, no significant change in cell cycle is seen for RKO cells exposed to *E. faecalis*-infected macrophages at an MOI of 1,000 (*right*). *B,* Co-culture with uninfected or *E. faecalis*-infected macrophages activates ATM/ATR DNA damage responses in HCT116 cells. Cyclin B1 is increased at 5, 10, and 24 hrs post-exposure to infected macrophages compared to uninfected controls. *C,* Laser scanning confocal microscopy shows γH2AX foci (*green*) in nuclei (*blue*) of HCT116 cells treated with supernatants from overnight cultures of *E. faecalis*-infected macrophages at an MOI of 1,000 (*lower panel*). Similar foci were rare for cells treated with supernatants from uninfected macrophages (*upper panel*). *D,* FACS analysis shows increased percentage of HCT116 cells (*left*) and RKO cells (*right*) with γH2AX foci following exposure to *E. faecalis*-infected macrophages at an MOI of 1,000 at 48 hrs compared to uninfected control (4.3% vs. 15.5% and 8.7% vs. 16.1%, respectively). Gray trace: untreated control; red trace: co-culture with uninfected control; green trace: co-culture with *E. faecalis*-infected macrophages.

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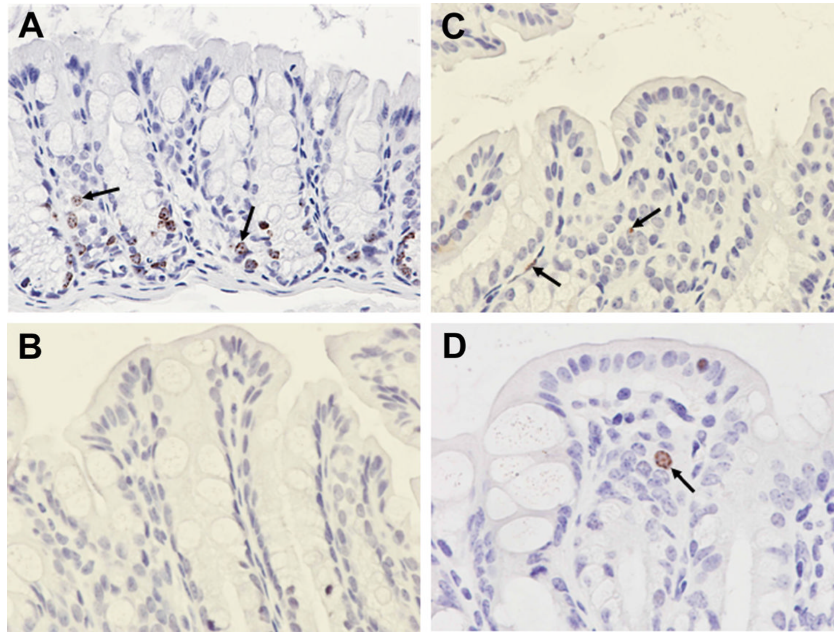

Figure 6. *E. faecalis* induces DSBs in vivo

A, Colons of wild-type mice show nuclei with numerous epithelial and stromal cells positive for γH2AX by immunohistochemistry following 13 Gy irradiation (arrows, 60×). *B,* Colonic mucosa from the ligation model for PBS controls show few nuclei staining for γH2AX (60×). *C,* Colonic mucosa from mice exposed to *E. faecalis* show numerous foci of nuclear staining for γH2AX in epithelial and stromal cells (arrows, 60×). *D,* Same as *C* showing nuclear γH2AX staining in stromal cell adjacent to colonic crypt (arrow, 60×).