TNF-α mediates macrophage-induced bystander effects through Netrin-1

Yonghong Yang1, 2, Xingmin Wang1, 2, Danny R. Moore1, 3, Stanley A. Lightfoot 4, and Mark M. Huycke1, 2, 3*

1The Muchmore Laboratories for Infectious Diseases Research, Research Service, Department of Veterans Affairs Medical Center, Departments of 2Radiation Oncology, 3Medicine, and 4Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

Running title: TNF-α mediates bystander effects

*Corresponding author: Mark M. Huycke, M.D.
Veterans Affairs Medical Center
921 N.E. 13th St.
Oklahoma City, OK 73104
E-mail: mark-huycke@ouhsc.edu
Phone: (405) 456-3306; Fax: (405) 456-1560

Key words: TNF-α, Tnfrsf1b, netrin-1; macrophage, tumorigenesis, colorectal cancer

Author Contributions: Mark M. Huycke and Yonghong Yang: study concept and design, analysis and interpretation of data, writing of the manuscript; Yonghong Yang, Xingmin Wang, Danny R. Moore: acquisition of data; Stanley A. Lightfoot: analysis and interpretation of data.

Conflicts of Interest: The authors disclose no conflicts.

Funding: Supported by National Institutes of Health grant CA127893 (to M.M.H.), the Oklahoma Center for the Advancement of Science and Technology grant HR10-032 (to X.W.), and funds from the Frances Duffy Endowment.

Word count (excluding abstract and references): 4,833

Number of figures: 7
Abstract

Macrophage-induced bystander effects have been implicated as an important mediator of chromosomal instability and colon cancer triggered by *Enterococcus faecalis*, a human intestinal commensal bacteria. There is little understanding about how inflammatory cytokines mediate bystander effects, but questions in this area are important because of the pivotal contributions made by inflammatory processes to cancer initiation and progression. Here we report that the central pro-inflammatory cytokine TNF-α acts as a diffusible mediator of the bystander effects induced by macrophages, an effect caused by a proliferation of macrophages that trigger epithelial cell production of Netrin-1, a neuronal guidance molecule. TNF-α-mediated bystander assays employed a murine co-culture system of primary colonic epithelial cells and *E. faecalis*-infected macrophages (in vitro), with an IL-10-deficient mouse model of colon cancer that involves long-term colonization with *E. faecalis* (in vivo). In cell co-cultures, we observed increased expression of the TNF-α receptor Tnfrsf1b and Netrin-1. These effects were blocked by anti-TNF-α antibody or by pretreatment with an inhibitor of NF-κB signaling. RNAi-mediated attenuation of Tnfrsf1b decreased TNF-α-induced netrin-1 production and augmented epithelial cell apoptosis in culture. Extending these observations, colon biopsies from *E. faecalis*-colonized IL-10-/- mice exhibited crypt hyperplasia and increased staining for macrophages, TNF-α, netrin-1, NF-κB, Tnfrsf1b and the proliferation marker PCNA, also displaying a reduction in epithelial cell apoptosis. Together, our results define a pathway for macrophage-induced bystander effects in which TNF-α triggers TNFRSF1b receptor signaling leading to increased production of Netrin-1, crypt hyperplasia and decreased epithelial cell apoptosis. In elucidating an important commensal-associated pro-inflammatory mechanism in the intestinal
microenvironment, our work highlights the role of Netrin-1 and a specific TNF-α receptor as candidate targets to prevent or treat colorectal cancer.

*Keywords:* CRC Model; Bystander effect; TNF-α; Netrin-1; Tnfrsf1b
Introduction

Accumulating evidence implicates intestinal commensals and pathogens as important triggers for epithelial cell transformation leading to colorectal cancer (1-5), a diagnosis with an overall lifetime risk of 5.3% for persons in the United States (6). *Enterococcus faecalis* is a common human intestinal commensal that produces DNA damage in epithelial cells and generates chromosomal instability through a macrophage-induced bystander effect (5, 7-9). The generation of extracellular superoxide by this microorganism—an unusual phenotype resulting from constrained respiration—substantially contributes to bystander effects and makes this commensal a useful model for studying bacterial mechanisms that initiate and/or promote colorectal cancer (5, 8, 10).

Bystander effects arising from bacterial activation of macrophages lead to aneuploidy, tetraploidy, and chromosomal instability in neighboring epithelial cells (5, 8, 9). This is analogous to observations by radiation biologists that irradiating myeloid cells or fibroblasts causes clastogens (*i.e.*, chromosome-breaking factors) to be produced that diffuse into neighboring unirradiated cells and cause CIN (11). This phenomenon is termed the radiation-induced bystander effect. In mice the prooxidant metabolism of *E. faecalis* can acutely activate nuclear factor (NF)-κB in colonic macrophages and induce a mucosal gene network associated with inflammation, apoptosis, and cell-cycle regulation (12). Changes in gene regulation are chronically sustained in interleukin (IL)-10 knockout mice colonized with *E. faecalis* where activated gene networks include tumor necrosis factor (TNF)-α, IL-1, interferon-γ, and IL-6 (13). The long-term result is colonic inflammation, dysplasia, and colorectal cancer (5, 13-15). Several enterococcal determinants have been implicated in the development of inflammation and cancer.
including an extracellular serine proteinase and extracellular superoxide production (5, 16). One mediator for macrophage-induced bystander effects has been identified, 4-hydroxy-2-nonenal, a mutagenic aldehyde produced by *E. faecalis*-infected macrophages and observed in the mucosa of colonized *Il10^-/-* mice (5). However, other potential mediators that may modulate proliferative or anti-apoptotic pathways and promote carcinogenesis remain uncharacterized.

A plausible mediator for macrophage-induced bystander effects is TNF-α. In murine models of colitis, TNF-α has been linked to tumorigenesis through NF-κB activation and induction of inflammation-associated cytokines and anti-apoptotic proteins (17-19). TNF-α is also known to promote carcinogenesis by damaging DNA, stimulating cancer growth, and promoting invasiveness (20-22). TNF-α signaling occurs through Tnfrsf1a (Tnfr1) and/or Tnfrsf1b (Tnfr2) receptors that, when bound, recruit intracellular adaptor proteins to activate multiple signal transduction pathways including NF-κB (23). Tnfrsf1b receptor signaling in particular has been linked to NF-κB activation in the azoxymethane-dextran sodium sulfate model of colitis-associated carcinogenesis (24). The potential relevance of such signaling in human colorectal cancer was recently noted in the Nurses’ Health Study cohort where surrogate markers for cytokine activation were assessed. In that population, baseline plasma TNFRSF1B (TNFR2), a marker of inflammation and soluble TNF antagonist, was significantly associated with an increased risk of human colorectal cancer (25), whereas no association was found for C-reactive protein or interleukin-6.

One mechanism by which TNF-α signaling and NF-κB activation could contribute to colorectal carcinogenesis is through the inhibition of apoptosis (20). One key regulator of apoptosis in the intestine is netrin-1. This diffusible protein was initially discovered as an
attractant for commissural axons (26). More recent data implicates netrin-1 and its dependence receptors in cancer initiation and progression (27). Many human cancers, including inflammation-associated colorectal cancer, are associated with upregulation of netrin-1 or loss of one or more of its cognate receptors (28-30). Increased binding of netrin-1 to dependence receptors—including deleted in colorectal cancer (DCC) and the UNC5H family members—inhibits p53-dependent apoptosis and promotes epithelial cell proliferation (27). The oncogenic properties of netrin-1 were noted in mice containing a germline mutation in $Apc^{+/1638N}$ that overexpressed netrin-1, and in an azoxymethane-dextran sodium sulfate model of colitis-associated carcinogenesis wherein netrin-1 binding was blocked using a protein fragment of DCC (29, 30). In both models, netrin-1 inhibited colonic epithelial cell apoptosis and promoted high grade adenomas with focal adenocarcinoma. In our work, we observed increased expression of $Ntn1$ in colonic epithelial cells both in vitro and in vivo using superoxide-producing $E.\ faecalis$ (12).

It remains to be determined whether TNF-$\alpha$ can mediate intestinal commensal-triggered bystander effects to modulate netrin-1 production and promote colorectal carcinogenesis. To further investigate this potential mechanism we measured TNF-$\alpha$ production by $E.\ faecalis$-infected macrophages and noted marked increases in vitro and in colonic macrophages from $Il10^/-$ mice colonized with $E.\ faecalis$. This finding was associated with colonic crypt hyperplasia and increased netrin-1 production. During the in vitro infection of macrophages by $E.\ faecalis$, inhibition of NF-$\kappa B$ or blocking Tnfrsf1b, but not Tnfrsf1a, abrogated this increase in netrin-1. Finally, Tnfrsf1b receptor signaling resulted in the activation of anti-apoptotic survival proteins. The pro-proliferative consequences of netrin-1 confirm TNF-$\alpha$ as an important mediator of macrophage-induced bystander effects. These findings substantially add to our understanding of
the mechanisms by which intestinal commensals contribute to autochthonous carcinogenesis in the colon.

**Materials and Methods**

**Cell lines and bacteria**

YAMC primary mouse colon epithelial cells (obtained from the Ludwig Institute for Cancer Research, New York in 2007). YAMC cells were last authenticated in 2011 by continuous growth at 33°C, senescence at 39.5°C, and PCR amplification of the transforming SV40 large T antigen. RAW264.7 cells were obtained from the American Type Culture Collection in 2003 and not further authenticated. Both cell lines were grown as previously described (8, 31). *E. faecalis* strain OG1RFSS was spontaneously derived from OG1RF—a protease-positive, Gram-positive human commensal—was grown in vitro as previously described (7, 8). A dual-chamber co-culture system for exposing YAMC cells to macrophages was used as previously described (8).

**Assay for TNF-α**

RAW264.7 murine macrophages were infected with sham or *E. faecalis* OG1RF at a multiplicity of infection (MOI) of 1000. Supernatants were collected over 72 hrs and TNF-α concentration determined using an ELISA kit according to the manufacturer’s instructions (eBioscience, San Diego, CA) with protein content normalized using recombinant mouse TNF-α (Cell Signaling Technology, Danvers, MA). NF-κB was irreversibly blocked using BAY11-7085
as a selective inhibitor of IκB-α phosphorylation (Santa Cruz Biotechnology, Santa Cruz, CA) (32).

**Gene silencing**

Gene expression was silenced using *Tnfrsf1b* siRNA with mouse specific reagents (Santa Cruz Biotechnology) or scrambled pooled non-targeting siRNA (Ambion, Austin, TX). Transient transfections were performed using lipofectamin 2000 reagent according to the manufacturer (Invitrogen, Camarillo, CA) and gene silencing confirmed by Western blot.

**Western blotting**

Protein extraction and Western blotting were performed as previously described using antibody to netrin-1 (Neuromics, Edina, MN); Tnfrsf1a, Tnfrsf1b, p-NF-κB p65, and cleaved caspase-3 (Cell Signaling Technology) (9). Murine β-actin antibody was purchased from BioVision (Milpitas, CA).

**RT-PCR**

Total RNA was extracted from YAMC cells using the NucleoSpin RNA II Kit (BD Biosciences, Palo Alto, CA) and cDNA synthesized using TaqMan Reverse Transcription Reagents according to manufacturer’s instructions (Applied Biosystems, Bedford, MA). Primers and PCR product sizes are listed in the Supplementary Table. PCR was performed by preheating to 94°C for 1 min followed by 30 cycles at 94°C for 20 sec, 56°C for 20 sec, and 72°C for 30 sec. PCR products were separated in a 2.5% agarose gel.
Apoptosis assay

Apoptosis was assessed by flow cytometry using the Annexin V FITC apoptosis detection kit according to the manufacturer’s instructions (EMD Biosciences) and quantified by flow cytometry (CellQuest Pro, San Jose, CA). The effect of TNF-α-induced netrin-1 expression on colonic epithelial cells was determined using YAMC cells after silencing Tnfrsf1b, or using cells treated with scrambled pooled non-targeting siRNA, 24 hrs following exposure to 100 ng/ml TNF-α. For each sample, >10,000 events were collected and groups compared using the Student’s t-test.

Colonization of Il10<sup>−/−</sup> mice

Conventionally housed Il10<sup>−/−</sup> mice develop aggressive enterocolitis (33). These same mice, when housed in specific-pathogen-free or germ-free environments show no pathology (34), with many commensals or mixtures of commensals failing to cause inflammation or cancer (34). Colonization with *E. faecalis* induces progressive colitis that results in colorectal cancer (14, 15). *E. faecalis* specifically triggers macrophages while many other intestinal commensals do not (5, 14, 15).

Specific pathogen-free Il10<sup>−/−</sup> knockout mice (C57BL, Jackson Laboratory, Bar Harbor, ME) were colonized with *E. faecalis* OG1RFSS as previously described (7). In brief, OG1RFSS expresses high-level resistance to spectinomycin and streptomycin (*i.e.*, >500 mg L<sup>−1</sup>). When these antibiotics are administered together in drinking water, sensitive intestinal gram-positive and gram-negative microaerophilic intestinal flora are suppressed permitting OG1RFSS to colonize. These antibiotics are inactive against anaerobes (that comprise >99% of the microbiota). The use of two antibiotics reduces the probability of spontaneous resistance or
superinfection by the minority population of microaerophilic intestinal microbiota. As these bacteria are sensitive to these antibiotics, the probability of spontaneous double-resistance events is extremely low (estimated at <10^{-12} per bacterium or <1 resistance event per 10^3 mice). This strategy, when coupled with contact barrier precautions and autoclaved food/water, permits long-term colonization. These antibiotics are not absorbed and so mice show no ill effect. The protocol was initiated for 10 week old male mice by the orogastric administration of OG1RFSS after 2 days on antibiotics. High density fecal colonization developed within 1 week at an average of 10^9 colony forming units per gram for mice receiving OG1RFSS. *E. faecalis* was never recovered from mice receiving sham. Colonization was checked every 8 weeks and at necropsy and remained unchanged. Colon biopsies were obtained at necropsy after 3 and 9 months of colonization. Crypt lengths in photomicrographs of colon sections were measured using a Photoshop CS3 ruler tool (Adobe, version 10.0.1) and converted to microns by magnification (100X). At least 50 crypts from three mice per group were analyzed. Protocols were approved by the University of Oklahoma Health Sciences Center and Oklahoma City Department of Veterans Affairs animal studies committees.

**Immunohistochemistry**

Immunohistochemical staining was performed as previously described (9). Primary antibodies included F4/80 and anti-mouse TNF-α (eBioscience); netrin-1, TNF-R1 (Tnfrsf1a), TNF-R2 (Tnfrsf1b), and PCNA (Santa Cruz Biotechnology); nuclear localization sequence (NLS)-specific anti-NF-κB/p65 (Rockland Immunochemicals, Gilbertsville, PA); and cleaved caspase-3 (Cell Signaling). Secondary antibodies were conjugated to horseradish peroxidase and
visualized using DAB Enhanced liquid substrate with nuclei counterstained using Mayer’s hematoxylin solution (Sigma, St. Louis, MO).

For Immunofluorescence tissue sections were incubated in 10% normal serum with 0.3 M glycine and 0.1% Tween in PBS for 30 min. A 1:50 dilution of anti-mouse TNF-α antibody and 1:100 dilution of anti-mouse F4/80 antigen FITC antibody were used as primary antibodies (eBioscience) and anti-rat IgG Texas Red (Santa Cruz Biotechnology) as secondary antibody. Nuclei were counterstained with 4,6-diamidino-2-phenylindole prior to laser-scanning confocal microscopy (Carl Zeiss Microscopy, Thornwood, NY).

**Statistical analysis**

Data were expressed as means with standard deviations. Student’s *t* test was used for comparison between experimental and control groups. *P* values less than 0.05 were considered statistically significant.

**Results**

**Macrophages produce TNF-α in *E. faecalis*-colonized *Il10*<sup>−/−</sup> mice**

TNF-α is a key mediator of inflammation that contributes to carcinogenesis through the activation of NF-κB (20, 22). To investigate whether TNF-α was involved in *E. faecalis*-triggered and macrophage-induced bystander effects, we noted, as expected, increased production of TNF-α by *E. faecalis*-infected macrophages (Supplementary Fig. S1). Untreated macrophages produced scant TNF-α. We colonized *Il10*<sup>−/−</sup> mice with *E. faecalis*, or administered sham, and biopsied colons after 3 and 9 months. We found inflammation and dysplasia in the
distal colons of colonized mice at 3 months and cancer at 9 months; no abnormal pathology was noted among shams. Using F4/80 staining, we found significantly increased numbers of macrophages in the lamina propria of colons compared to shams. For colonized mice, these same cells showed intense staining for TNF-α compared to sham ($P < 0.001$, Fig. 1A, 1B, and Supplementary Fig. S2). Immunofluorescence confirmed TNF-α production in lamina propria macrophages (Fig. 1C), suggesting activation of these cells by colonizing *E. faecalis*.

**E. faecalis-infected macrophages induce netrin-1**

To explore mechanisms by which TNF-α might contribute to bystander effects, we measured netrin-1 production in YAMC epithelial cells treated with TNF-α. We noted substantial increases after only 5 hrs that persisted through 72 hrs (Fig. 2A). Similar results were found following exposure of these cells to *E. faecalis*-infected macrophages in the dual-chamber co-culture system (Fig. 2B). Increases in netrin-1 occurred in a dose-dependent fashion except for the highest TNF-α concentration tested (Fig. 2C). Finally, production of netrin-1 by YAMC cells increased following exposure to greater numbers of infected macrophages (Fig. 2D). RT-PCR demonstrated increased *Ntn1* expression by TNF-α and *E. faecalis*-infected macrophages (Supplementary Fig. S3A-D). Colon biopsies from *E. faecalis* colonized *Il10<sup>-/-</sup>* mice showed intense staining for netrin-1 in epithelial cells compared to sham (Fig. 2E and Supplementary Fig. S4), and were consistent with in vitro results obtained using YAMC cells exposed to *E. faecalis*-infected macrophages.

**TNF-α induces netrin-1 production**
To confirm these results, we measured netrin-1 production by YAMC epithelial cells that had been treated with TNF-α and anti-TNF-α antibody or heat-denatured TNF-α. Both controls led to significantly decreased netrin-1 production (Fig. 3A). Anti-TNF-α antibody blocked netrin-1 production in a dose-dependent manner (Fig. 3B). Of note, the scant netrin-1 produced by untreated YAMC cells that was blocked by anti-TNF-α antibody (Supplementary Fig. S5), suggesting in vitro autocrine production of TNF-α by YAMC cells. Finally, in the dual-chamber co-culture system, this antibody was able to completely block netrin-1 production in YAMC cells exposed to *E. faecalis*-infected macrophages (Fig. 3C), confirming specificity for this cytokine on netrin-1 production via a bystander effect.

**TNF-α promotes netrin-1 via NF-κB**

 Activation of NF-κB contributes to tumor formation, at least in part, by inducing anti-apoptotic factors in epithelial cells. To establish whether netrin-1 was induced through NF-κB activation, we exposed YAMC cells with an irreversible inhibitor of NF-κB and followed by TNF-α treatment. After 24 hrs, netrin-1 significantly decreased (Fig. 4A), a finding in agreement with previous observations showing *Ntn1* as an NF-κB target (28). Similar results were seen using the dual-chamber co-culture system (Fig. 4B). We next performed immunohistochemical staining on colon sections from Il10⁻/⁻ mice colonized with *E. faecalis* for 3 or 9 months using antibody to the nuclear localization signal of p65 (RelA). Lamina propria macrophages and epithelial cells stained intensely for this marker compared to sham (Fig. 4C and Supplementary Fig. S6), a finding consistent with the concept that NF-κB activation results in TNF-α production by colonic macrophages and, via bystander effects, increased netrin-1 production by epithelial cells.
**TNF-α induces Tnfrsf1b**

The activation of NF-κB in intestinal epithelial cells by TNF-α through Tnfrsf1b receptor signaling has been previously linked to carcinogenesis (24). To determine whether TNF-α binding to epithelial cells was involved in increased netrin-1 production, we initially assayed YAMC cells for Tnfrsf1a and Tnfrsf1b and noted strong induction of Tnfrsf1b by TNF-α (Fig. 5A and B and Supplementary Fig. S3A and C). In contrast, expression of Tnfrsf1a was slightly reduced by treatment with TNF-α. Similarly, Tnfrsf1b production increased in YAMC cells co-cultured with *E. faecalis*-infected macrophages while Tnfrsf1a only slightly increased (Fig. 5C and D). A similar change, however, was not seen in mRNA (Supplementary Fig. 3B and D), suggesting post-transcriptional modifications of this receptor due to TNF-α and/or other cytokines from *E. faecalis*-infected macrophages. Induction of Tnfrsf1b was specific for TNF-α as anti-TNF-α antibody, or heat-inactivated TNF-α, blocked the increase Tnfrsf1b expression (Fig. 5E). Colon biopsies from *Il10*−/− mice colonized with *E. faecalis* confirmed these *in vitro* findings. Increased staining for Tnfrsf1b was noted in colonic epithelial cells compared to sham while there were no changes in Tnfrsf1a staining (Fig. 5F). These results are consistent with colonization by *E. faecalis* causing enhanced Tnfrsf1b receptor expression on colonic epithelial cells.

**Tnfrsf1b promotes TNF-α-induced apoptosis**

The primary mechanism by which netrin-1 promotes colorectal carcinogenesis is through the pro-proliferative modulation of apoptosis (29, 30, 35). To establish whether TNF-α signaling through Tnfrsf1b receptors was responsible for the enhanced production of netrin-1 by epithelial cells, we silenced *Tnfrsf1b* in YAMC cells using *Tnfrsf1b*-specific siRNA. TNF-α treatment of
these cells showed a 60% decrease in Tnfrsf1b expression and concomitant 40% decrease in netrin-1 compared to cells transfected with non-targeting siRNA (Fig. 6A). This observation confirmed the predominant role of Tnfrsf1b receptor in TNF-α-induced netrin-1 production for these primary colonic epithelial cells.

We next determined the effect of Tnfrsf1b receptor signaling on apoptosis in YAMC cells. Tnfrsf1b-silenced YAMC cells were stained with annexin V-FITC. As shown in Fig. 6B, following treatment with TNF-α, the percentage of apoptotic cells significantly increased for Tnfrsf1b-silenced cells compared to cells transfected with non-targeting siRNA (31.9 ± 2.0 vs. 8.2 ± 0.7, P < 0.01), supporting induction of netrin-1 by TNF-α through the action of Tnfrsf1b. Notably, the percentage of apoptotic cells for Tnfrsf1b-silenced cells also increased in the absence of TNF-α compared to cells transfected with non-targeting siRNA (19.7 ± 1.2 vs. 6.4 ± 3.2, P < 0.05). This might be due to scant production of TNF-α by YAMC cells (Supplementary Fig. S5).

Finally, to investigate whether netrin-1 contributed to cellular proliferation through anti-apoptotic signaling, we measured p-NF-κB (p65) and cleaved caspase-3 in Tnfrsf1b-silenced YAMC cells. We found decreased p-NF-κB (p65) in Tnfrsf1b-silenced cells compared to cells transfected with non-targeting siRNA and no additional induction by TNF-α, implying impaired NF-κB activation when Tnfrsf1b was silenced. Moreover, we noted increased cleaved caspase-3 compared to cells transfected with non-targeting siRNA and additional increases when Tnfrsf1b-silenced cells were treated with TNF-α (Fig. 6C). We next stained colon crypts from E. faecalis colonized Il10−/− mice for cleaved caspase-3 and noted marked decreases in staining at the tops of crypts (Fig. 6D). In addition, PCNA staining was increased in colonic epithelial cells (Fig. 6E).
Finally, these pro-proliferative effects were associated with colon crypt hyperplasia as measured by increased crypt length in mice colonized with *E. faecalis* for 3 and 9 months compared to sham (Fig. 6F).
Discussion

Substantial credible evidence identifies inflammation as pivotal to the initiation and promotion of many common tumors including colorectal cancer (17, 18). However, interactions among diverse factors involved in colorectal carcinogenesis, including host determinants (e.g., age and genetic predisposition), dietary composition, intestinal microbiota, and pharmacological intervention, have challenged mechanistic studies in this field. Recently, we used \( IL10^{-/-} \) mice colonized with \( E.\ faecalis \) to investigate bystander effects as a model for endogenous (or autochthonous) carcinogenesis triggered by a human commensal (5, 8, 9). In this model, local inflammation generates chromosomal instability and promotes colorectal cancer through bystander effects.

\( E.\ faecalis \) is particularly relevant to the study of colon carcinogenesis because the microorganism belongs to a small number of human commensals, including \( E.\ coli \) and \( Bacteroides\ fragilis \), that promote colorectal adenomas and cancer in murine models (3, 4, 15). \( IL-10^{-/-} \) mice are attractive for modeling bacterial triggers because loss of this cytokine allows colonic macrophages to be activated by the intestinal microbiota (33, 36). These triggers, however, appear to be highly specific with pathology only developing with \( E.\ faecalis \) or \( E.\ coli \) are used and not with other members of the pathogen-free flora (34, 37). Finally, the IL-10 knockout model avoids potent exogenous carcinogens (\( e.g. \), azoxymethane) and allows dietary control. One potential limitation for conventionally housed \( IL10^{-/-} \) mice colonized with \( E.\ faecalis \) is the need to use antibiotics to maintain high-density colonization. Without such pressure, exogenous \( E.\ faecalis \) only transiently colonizes mice. Potential side-effects, however, are
minimized by using oral non-absorbable antibiotics and are further controlled by treating shams similarly.

Our study further expands the conceptual basis for macrophage-induced bystander effects. This phenomenon was initially observed by radiation biologists who found chromosomal instability in non-irradiated cells exposed to diffusible mediators from irradiated cells (11). Microbeam irradiation experiments, where only the cellular cytoplasm is targeted, clearly demonstrate that DNA damage is not necessary to generate these diffusible mediators. The first reports were from studies of individuals exposed to low-linear-energy transfer whole body irradiation (e.g., x-rays) (38, 39). High linear-energy-transfer radiation (e.g., α-particles), however, appears more efficient at producing mediators. Bystander effects occur in vivo as has been confirmed in experiments using syngenic mice (40, 41). Finally, both radiation-induced and *E. faecalis*-triggered bystander effects depend on cyclooxygenase-2 (8, 42), and may well provide insights into the established role for this enzyme in carcinogenesis (43).

TNF-α and NF-κB are key contributors to inflammation-associated tumorigenesis (20, 44). In the *Mdr2*-knockout model of hepatocellular carcinoma, TNF-α activates NF-κB and is essential for tumor formation (20). Similarly, blocking TNF-α in a carcinogen-colitis model of colorectal cancer reduces tumor formation (19). TNF-α may contribute to carcinogenesis by inducing apoptosis. In our system, TNF-α suppressed apoptosis in YAMC cells through Tnfrsf1b receptor signaling. Although we saw markedly increased Tnfrsf1b receptor expression in the colonic epithelial cells of *E. faecalis* colonized *Il10*−/− mice, a direct linkage of TNF-α signaling through these receptors awaits further investigation. NF-κB in epithelial cells was activated in vitro and in vivo by *E. faecalis*-triggered (and TNF-α-mediated) bystander effects. We found that
NF-κB led to induction of netrin-1, a known transcriptional target for this pleiotrophic transcription factor (30). These findings are also consistent with previous results that show netrin-1 is acutely upregulated by superoxide-producing *E. faecalis* (12).

Netrin-1 is an oncogene that is overexpressed in 70% of colorectal adenocarcinomas from patients with inflammatory bowel disease (30). Approximately one-quarter of sporadic colorectal cancers overexpress netrin-1 (28, 30). The loss of netrin-1 receptors that act as tumor suppressors, however, is more common and found in the majority of these cancers. We noted that *E. faecalis*-infected macrophages specifically induced netrin-1 in a time- and dose–dependent fashion and, compared to shams, was visible by immunostaining in the colonic epithelial cells of *Il10*−/− mice colonized with *E. faecalis*. These findings indicate that, in addition to 4-hydroxy-2-nonenal, a known mutagen and spindle poison (5), TNF-α also contributes to macrophage-induced bystander effects via the anti-apoptotic action of netrin-1.

The contributions of TNF-α toward carcinogenesis are multifactoral and include initiation and promotion of malignant cells, development of angiogenesis, and modulation of adaptive immunity including responses to hormones and chemotherapeutics (18, 20-22, 44). Many of these cellular events are orchestrated by NF-κB. We investigated signaling pathways for TNF-α as a potential mediator of bystander effects and noted that anti-TNF-α antibody blocked the nuclear localization of NF-κB/p65. In addition, the NF-κB inhibitor Bay11-7085 decreased netrin-1, presumably by blocking TNF-α-induced phosphorylation of IκBα (32). These findings agree with previous reports that show NF-κB drives netrin-1 expression (28).

TNF-α can bind at least two receptors on colonic epithelial cells. Tnfrsf1a receptors possess a cytoplasmic death domain that rapidly engages apoptotic signaling, whereas Tnfrsf1b
receptors recruit adaptor proteins that activate NF-κB and promote gene transcription to protect against apoptosis. The importance of TNFRSF1B in carcinogenesis was recently noted in a large cohort study where cleaved soluble receptor was associated with an increased risk for colorectal cancer (25). We investigated changes in Tnfrsf1a and Tnfrsf1b on YAMC cells after treatment with TNF-α and found that only Tnfrsf1b was induced. Similarly, *E. faecalis* colonization of *Il10−/−* mice only appeared to increase Tnfrsf1b expression on colonic epithelial cells. For the macrophage-induced bystander effect, Tnfrsf1b receptor signaling was specific for netrin-1 since silencing *Tnfrsf1b* decreased production and increased apoptosis. However, we cannot entirely exclude the possibility that increased apoptosis may have resulted from pro-apoptotic signaling through Tnfrsf1a receptors.

In this study we found that TNF-α acted as a diffusible mediator for macrophage-induced bystander effects (Fig. 7). This inflammatory cytokine was triggered by *E. faecalis* and led to colonic epithelial cell proliferation through the anti-apoptotic action of netrin-1 (35). Netrin-1 was up-regulated through Tnfrsf1b receptor signaling and activation of NF-κB. Netrin-1 is an anti-apoptotic ligand that binds DCC and the UNC5H family of dependence receptors and in murine models promotes progression of colon adenomas to high grade cancers (29, 30). In conclusion, our findings show that TNF-α is an important mediator for macrophage-induced bystander effects. Through Tnfrsf1b receptor signaling initiated by *E. faecalis*-infected macrophages, TNF-α leads to increased epithelial cells production of netrin-1 causing colonic crypt hyperplasia and a reduction in epithelial cell apoptosis. TNF-α is a diffusible mediator for macrophage-induced bystander effects in the colon and provides additional potential targets for the prevention of colorectal cancer.
Acknowledgments

We thank Jim Henthorn in the Flow Cytometry Laboratory at the University of Oklahoma Health Sciences Center and Robert Whitehead at Vanderbilt University and Ludwig Institute for their gift of YAMC cells.
References


Figure Legends

Figure 1. E. faecalis-infected macrophages produce TNF-α. A, Immunohistochemical staining of colons from Il10−/− mice colonized with E. faecalis for 9 months; increased numbers of macrophages (F4/80 positive, brown) and B, staining for TNF-α (brown) are noted in the lamina propria (40X), but not seen in shams. Numbers of F4/80 and TNF-α-positive cells per 20X field are increased in colons from Il10−/− mice colonized with E. faecalis for 9 months compared to sham (***P < 0.001). C, Immunofluorescence staining of colon biopsies from E. faecalis colonized Il10−/− mice using F4/80 (red) and TNF-α (green) confirm co-localization of TNF-α to macrophages (yellow).

Figure 2. TNF-α increases netrin-1. A, YAMC cells increase netrin-1 production when treated with 100 ng/ml TNF-α. B, In the dual-chamber co-culture system, E. faecalis-infected macrophages show peak production of netrin-1 at 48-72 hrs. C, Netrin-1 production increases with higher concentrations of TNF-α except at 500 ng/ml when inhibition is noted. D, E. faecalis-infected macrophages similarly increase netrin-1 in a dose-dependent fashion. E, Immunohistochemical staining shows increased netrin-1 in colonic epithelial cells for Il10−/− mice colonized with E. faecalis for 9 months compared to sham (40X).

Figure 3. Anti-TNF-α blocks TNF-α-induced netrin-1 production. A, Treatment of YAMC cells with murine TNF-α increases netrin-1 production; heat-inactivation of TNF-α and anti-TNF-α antibody block netrin-1 compared to control. B, Anti-TNF-α antibody blocks netrin-1 by YAMC cells in a dose-dependent manner. C, In the dual-chamber co-culture system, increasing anti-TNF-α antibody similarly blocks netrin-1 production by YAMC cells exposed to E. faecalis-infected macrophages cells.
Figure 4. TNF-α induces netrin-1 production via NF-κB activation. A, TNF-α-induced netrin-1 decreases in YAMC cells at 24 hrs when pre-treated with Bay11-7085; NF-κB activation decreases using antibody to nuclear localization signal of NF-κB/p65. B, Similarly, Bay11-7085 blocks netrin-1 in YAMC cells exposed to E. faecalis-infected macrophages using the dual-chamber co-culture system. C, Colon macrophages from Il10-/- mice colonized with E. faecalis for 9 months (bottom, inset, green arrows, 40X) stain intensely for nuclear localization sequence of NF-κB/p65 (20X); epithelial cells also stain strongly (bottom, inset, red arrows); in contrast, weak or no staining is seen for sham-treated mice (top).

Figure 5. TNF-α increases Tnfrsf1b receptors. A, Expression of Tnfrsf1b receptors increases in YAMC cells at 100 ng/ml TNF-α at 24, 48, and 72 hrs whereas Tnfrsf1a receptors are slightly reduced. B, At 48 hrs, TNF-α concentrations ≤100 ng/ml increase Tnfrsf1b in YAMC cells in a dose–dependent fashion. No significant increases are seen for Tnfrsf1a. C, Tnfrsf1b production increases for YAMC cells co-cultured with E. faecalis-infected macrophages at 24, 48, and 72 hrs. D, At 48 hrs, Tnfrsf1b production increases in YAMC cells co-cultured with E. faecalis-infected macrophages in a dose-dependent manner. E, Heat-inactivated TNF-α and anti-TNF-α antibody block TNF-α induction of Tnfrsf1b compared to control. F, Immunohistochemical staining shows increased Tnfrsf1b in colonic epithelial cells from Il10-/- mice colonized with E. faecalis for 9 months compared to sham; no change is seen for Tnfrsf1a (20X).

Figure 6. TNF-α signaling through Tnfrsf1b blocks apoptosis and promotes proliferation. A, Tnfrsf1b receptors and netrin-1 are reduced 60% and 40% (**P < 0.01), respectively, in YAMC cells following transfection with Tnfrsf1b-specific siRNA (si-Tnfrsf1b) compared to non-targeting siRNA (si-Ctrl) in the presence of 100 ng/ml TNF-α. B, Flow cytometry shows
increased numbers of apoptotic cells for \textit{Tnfrsf1b}-silenced YAMC cells compared to cells transfected with non-targeting siRNA; the proportion of apoptotic YAMC cells increases at 24 hrs following treatment with 100 ng/ml TNF-\(\alpha\) for \textit{Tnfrsf1b}-silenced cells compared to cells transfected with non-targeting siRNA. \textit{C}, Phosphorylated NF-\(\kappa\)B (p65) decreases in \textit{Tnfrsf1b}-silenced cells compared to cells transfected with non-targeting siRNA. No increase in p-NF-\(\kappa\)B (p65) is seen in these cells following treatment with TNF-\(\alpha\). Cleaved caspase-3 increases in \textit{Tnfrsf1b}-silenced cells compared to cells transfected with non-targeting siRNA and with additional increases following treatment with 100 ng/ml TNF-\(\alpha\). \textit{D}, Immunohistochemical staining shows decreased cleaved caspase-3 at the tops of colonic crypts (\textit{arrows}) from colon sections for \textit{Il10}^{-/-} mice colonized with \textit{E. faecalis} for 9 months compared to sham (20X). \textit{E}, PCNA staining increases in epithelial cells for \textit{Il10}^{-/-} mice colonized with \textit{E. faecalis} for 9 months compared to sham (20X). \textit{F}, Colon crypt length increases for mice colonized with \textit{E. faecalis} at 3 (\textit{blue}) and 9 months (\textit{red}) compared to sham (***\(P < 0.001\)).

\textbf{Figure 7. TNF-\(\alpha\) promotes netrin-1 through bystander effects.} \textit{A}, Normal colon crypt: stem cells produce the terminally differentiated epithelial cells within a crypt; netrin-1 is constitutively produced by epithelial cells at crypt base and blocks apoptosis until cells migrate to the top of the crypt; macrophages are quiescent in the absence of a specific bacterial trigger. \textit{B}, In \textit{Il10}^{-/-} mice, colonizing \textit{E. faecalis} translocate intact epithelium (presumably through M cells as overlying mucus poses a barrier to commensal bacterial interaction with epithelial cells) and activates tissue macrophages to produce TNF-\(\alpha\). \textit{C}, Inset of epithelial cell: TNF-\(\alpha\) mediates bystander effects by binding \textit{Tnfrsf1b} receptors on epithelial cells to activate NF-\(\kappa\)B and increase netrin-1; netrin-1 acts in an autocrine and/or paracrine fashion to bind dependence receptors (e.g., DCC or UNC5H) and provides anti-apoptotic signals for epithelial cells resulting in elongated crypts.
Figure 2
Figure 3

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Netrin-1</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (100 ng/ml)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Heat-inactivated TNF-α</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anti-TNF-α antibody (100 ng/ml)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Anti-TNF-α antibody (ng/ml)</th>
<th>Netrin-1</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Anti-TNF-α antibody (ng/ml)</th>
<th>E. faecalis-infected MΦ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5

A

B

C

D

E

F

Sham

E. faecalis

TNF-α (100 ng/ml)  
Heat-inactivated TNF-α  
Anti-TNF-α antibody (100 ng/ml)
Figure 6

A: TNF-α (100 ng/ml) dosage and si-Tnfrsf1b or si-Ctrl treatments

B: Percentage of apoptotic cells under different conditions

C: si-Tnfrsf1b or si-Ctrl treatments

D: Cleaved caspase-3 activity assessed by western blotting

E: PCNA expression in Sham and E. faecalis groups

F: Crypt length comparison between Sham and OG1RFSS groups

**P < 0.01
***P < 0.001