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ARTICLE

Rifaximin decreases virulence of Crohn's disease-associated *Escherichia coli* and epithelial inflammatory responses

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Abstract

Escherichia coli with an adherent and invasive pathotype (AIEC) is implicated in the pathogenesis of Crohn's disease (CD). Rifaximin improves symptoms in mild-to-moderate CD. It is unclear if this outcome is due to its effects on bacteria or intestinal epithelial inflammatory responses. We examined the effects of rifaximin on the growth and virulence of CD-associated *E. coli* and intestinal epithelial inflammatory responses. Seven well-characterized CD-associated *E. coli* strains (six AIEC, one non-AIEC; four rifaximin-resistant, three sensitive) were evaluated. We assessed the effects of rifaximin on CD-associated *E. coli* growth, adhesion to, and invasion of epithelial cells, virulence gene expression, motility, and survival in macrophages. Additionally, we determined the effects of rifaximin on intestinal epithelial inflammatory responses. In vitro rifaximin exerted a dose-dependent effect on the growth of sensitive strains but did not affect the growth of resistant strains. Rifaximin reduced adhesion, invasion, virulence gene expression and motility of CD-associated *E. coli* in a manner that was independent of its antimicrobial effect. Furthermore, rifaximin reduced IL-8 secretion from pregnane X receptor-expressing T84 colonic epithelial cells. The effect of rifaximin on adhesion was largely attributable to its action on bacteria, whereas decreases in invasion and cytokine secretion were due to its effect on the epithelium. In conclusion, our results show that rifaximin interferes with multiple steps implicated in host-AIEC interactions related to CD, including adhesion to, and invasion of epithelial cells, virulence gene expression, motility, and pro-inflammatory cytokine secretion. Further study is required to determine the relationship of these effects to clinical responses in CD patients.

Introduction

Inflammatory bowel disease (IBD) is a group of chronic inflammatory gastrointestinal disorders most prevalent in Europe and North America [1, 2]. The most common types of IBD are Crohn's disease (CD), a transmural granulomatous disease that most frequently involves the ileum and ulcerative colitis (UC) [1].

Mounting evidence supports a pivotal role for the enteric microflora in the initiation and perpetuation of IBD, with selective alterations in the microbiome (dysbiosis) exploiting genetic defects in innate immunity associated with CD [3–5]. Dysbiosis, characterized by an increase in Enterobacteriaceae, including *Escherichia coli* (*E. coli*) with an adherent and invasive pathotype (AIEC), has been consistently linked to ileal CD [4, 6, 7]. AIEC display pathogen-like behavior when incubated with cultured cells and resemble extra intestinal pathogenic *E. coli* (ExPEC) in genotype and virulence [4]. Acute inflammation triggers the proliferation of AIEC [8], and AIEC can utilize inflammation-related substrates for growth [9]. Concordant induction of flagellae (FliC) and fimbrial adhesins (e.g., FimH, LpFA) is thought to enable AIEC to invade or translocate across the intestinal mucosa [9, 10], enabling access to macrophages, in which AIEC can persist and proliferate to promote an escalating cycle of inflammation and AIEC colonization [10].

Belgin Dogan and Jing Fu contributed equally to this work.

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Table 1 Characteristics of the *E. coli* strains used in this study

Strain ID	Disease	Patient treated with rifaximin	Rifaximin resistance	MIC (mg/liter)	Phylotype	AIEC	Reference
524-2	CD	Yes	R	> 1024	B1	Y	[5, 14]
24LW-1	CD	Yes	R	> 1024	A	Y	[14]
578-1	CD	Yes	R	> 1024	D	Y	[5, 14]
T75	CD	Yes	R	> 1024	A	N	[5, 14]
541-15	CD	No	S	32	A	Y	[5, 14]
541-1	CD	No	S	32	B1	Y	[5, 14]
LF82	CD	ND	S	32	B2	Y	[7]

R: Resistant, *S*: Sensitive

The consistent association of dysbiosis and *E. coli* with CD suggests that antibiotics directed against aberrant microbiota could be effective in resolving CD. This notion is supported by the ability of rifaximin, a non-absorbable derivative of rifampin with antimicrobial activity against Gram-positive and Gram-negative, aerobic and anaerobic flora including *E. coli* [11], to improve symptoms in patients with mild-to-moderate CD [11–13]. The identification of *E. coli* with resistance to rifaximin in ileum of CD patients treated with rifaximin indicates that the outcome of treatment could be impacted by antimicrobial resistance [14]. However, rifaximin has also been shown to reduce the symptoms of enteric infections without changing the overall composition of the gut microbiota [15–17]. These observations suggest that the beneficial effects of rifaximin might be independent of its ability to kill bacteria, potentially mediated by an effect on virulence traits, that have been described for diarrheagenic *E. coli* and *Shigella* [18–20]. It is against this background that we sought to examine the effects of rifaximin on the growth and virulence of CD-associated AIEC that are sensitive and resistant to rifaximin and intestinal epithelial inflammatory responses.

Materials and methods

Bacterial strains and growth conditions

Seven well-characterized CD-associated *E. coli* strains (six AIEC, one non-AIEC; four rifaximin-resistant MIC of > 1024 µg/ml, and three sensitive MIC of < 32 µg/ml) were evaluated (Table 1). Rifaximin-resistant *E. coli* were isolated from CD patients who were treated with rifaximin. Unless stated otherwise rifaximin-resistant and sensitive strains were grown in Luria–Bertani (LB) broth containing 128 µg/ml and 8 µg/ml rifaximin, respectively. Rifaximin was provided by Alfa Wassermann (Bologna, Italy) and was dissolved in DMSO at a stock concentration of 50 mg/ml. DMSO was added to growth or cell culture media at a volume equivalent to the volume used to deliver rifaximin as a vehicle control.

Cell lines and culture conditions

Human colonic epithelial cell lines Caco-2 (ATCC HTB-37) and T84 (ATCC CCL-248), and the murine macrophage cell line J774A.1 (ATCC TIB-67) were obtained from American Type Culture Collection. The HCT116 cell line was kindly provided by Craig Stevens (University of Edinburgh). All cell lines were grown according to ATCC procedure.

Bacterial growth

E. coli were grown in LB broth until mid-log phase and were diluted to 5×10^5 CFU/ml in fresh LB broth containing 2, 4, 8, 16, 32, 64, 128, 256, 512 µg/ml rifaximin or DMSO. Bacteria were grown at 37 °C shaking for 24 h. Standardized growth curve analysis was performed using a Bioscreen C automated plate reader (Growth Curves USA, NJ) by measuring OD_{600}. All experiments were completed in triplicate and performed twice.

Epithelial cell adhesion and invasion

For adhesion and invasion assays cells and bacteria were pre-incubated with or without rifaximin containing media prior to assay. Rifaximin was maintained in cell culture media during the infection period. At a rifaximin concentration of 128 µg/ml, the concentration at which resistant bacteria were grown, epithelial cells detached from the culture plates during overnight pre-incubation period. This effect was absent when cells were pretreated with rifaximin at 32 µg/ml overnight (Supplementary Fig. S1). In experiments with rifaximin-sensitive strains, cells were pretreated with rifaximin at 8 µg/ml, unless stated otherwise, to reduce the chance of rifaximin diffusing into the host cells. Caco-2, T84, or HCT116 cells were seeded in 24-well plates (Caco-2 and HCT116 cells at 2×10^5 cells/well and T84 cells at

5×10^5 cells/well). The next day cells were pre-treated with rifaximin (resistant strains, 32 μg/ml; sensitive strains, 8 μg/ml) or vehicle control for 18 h. *E. coli* cultures were grown in LB broth in the presence of rifaximin (resistant strains, 128 μg/ml; sensitive strains, 8 μg/ml) or vehicle control. After an overnight incubation at 37 °C, bacteria were diluted in cell culture media (1×10^7 CFU/ml) with or without rifaximin (resistant strains: 64 μg/ml or vehicle control; sensitive strains: vehicle control). Epithelial cell counts and viability were determined before and after each experiment by trypan blue exclusion to ensure equal cell numbers of viable cells in vehicle control and rifaximin treated groups. Epithelial cells were infected with 1×10^7 CFU/well bacteria for 3 h. For adhesion assays, cells were washed three times with PBS 3 h after infection, then lysed with 1% triton in H_2O for 10 min. The total number of CFU recovered per well was determined by plating serial dilutions on LB agar.

For invasion assays, cells were washed three times with PBS 3 h after infection and treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. Bacterial numbers were determined as described above. Final results were expressed as the ratio of adhesion or invasion of *E. coli* with rifaximin to that of *E. coli* with vehicle control. Each experiment was performed in triplicate and repeated three times.

Giemsa staining

Adherent bacteria were visualized by Giemsa staining. Cells were fixed with methanol for 5 min, air-dried, stained with Giemsa (diluted 1:20 in H_2O) for 30 min, rinsed extensively with water and air dried. Multiple images were acquired with a cell imager (ZOE™, BIO-RAD) for two independent experiments.

RNA isolation and quantitative real-time PCR

The effect of rifaximin on the expression of 12 genes associated with AIEC motility, adhesion to, and invasion of epithelial cells, M cell translocation, iron acquisition and survival in macrophages [9, 10, 21–25] was determined using quantitative real-time PCR (qRT-PCR) with the primers listed in Supplementary Table S1.

RNA was extracted from mid-log phase cultures grown in LB broth in the presence of rifaximin (resistant strains, 128 μg/ml; sensitive strains, 8 μg/ml) or vehicle control, using the Qiagen RNA Protect-RNeasy Kit. DNA-free RNA was used for two-step qRT-PCR reactions, using Qiagen's QuantiTect Reverse Transcription Kit and QuantiNova SYBR Green PCR Kit. qRT-PCR was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The comparative quantification (ΔCt) method was used to determine up–regulation or down-regulation. The average cycle threshold (Ct) value from each sample in triplicate was obtained for both target and housekeeping (*mdH*) genes. The average Ct of a target gene was subtracted from the average Ct of the housekeeping gene to obtain ΔCt, or ΔCt^R for rifaximin-treated samples. The change of Ct values of a targeted gene in the presence and absence of rifaximin was calculated as ΔΔCt (ΔCt^R–ΔCt). Finally the relative quantity (RQ) was calculated by using the equation $RQ = 2^{-\Delta\Delta Ct}$.

Motility

The effect of rifaximin on *E. coli* motility was evaluated in soft agar. *E. coli* was grown overnight at 37 °C in LB broth with rifaximin (resistant strains: 128 μg/ml, sensitive strains: 8 μg/ml) or vehicle control. Two-μl of overnight culture was inoculated onto soft agar (1% tryptone, 0.5% NaCl, 0.25% agar) containing rifaximin (128 μg/ml for resistant strains and 8 μg/ml for sensitive strains) or vehicle control, and plates incubated at 30 °C overnight. Motility was assessed by measuring the diameter of the circle formed by each strain in the absence and presence of rifaximin. Non-motile *E. coli* strain T75 was excluded from analysis.

Survival in macrophages

J774 murine macrophages were seeded in 24-well plates at 2×10^5 cells/well and grown for 1 day at 37 °C with 5% CO_2. *E. coli* was grown in LB broth with rifaximin (resistant strains: 128 μg/ml; sensitive strains: 8 μg/ml rifaximin) or vehicle control overnight at 37 °C. Bacteria were diluted in cell culture media (4×10^6 CFU/ml) with or without rifaximin (resistant strains: 32 μg/ml rifaximin or vehicle control; sensitive strains: no rifaximin). J774 cells were infected with bacteria for 1 h then washed three times with PBS and treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. After 1 h the gentamicin concentration was reduced to 20 μg/ml to limit gentamicin diffusion into macrophages. Rifaximin or vehicle control (resistant strains 32 μg/ml rifaximin; sensitive strains 4 μg/ml rifaximin) was added to the cell culture media. For survival in macrophages the number of bacteria was determined at 1 and 24 h post-gentamicin by lysing cells in 1% Triton X-100 and plating serial dilutions. Survival was expressed as the mean percentage of the number of bacteria recovered after 1 h post-infection, defined as 100%. Final results were expressed as the ratio of persistent *E. coli* with rifaximin to that of *E. coli* without rifaximin.

Cytokine secretion

The production of IL-8, IL-12 (p40), and IL-6 in response to *E. coli* ± rifaximin was determined by ELISA (Invitrogen).

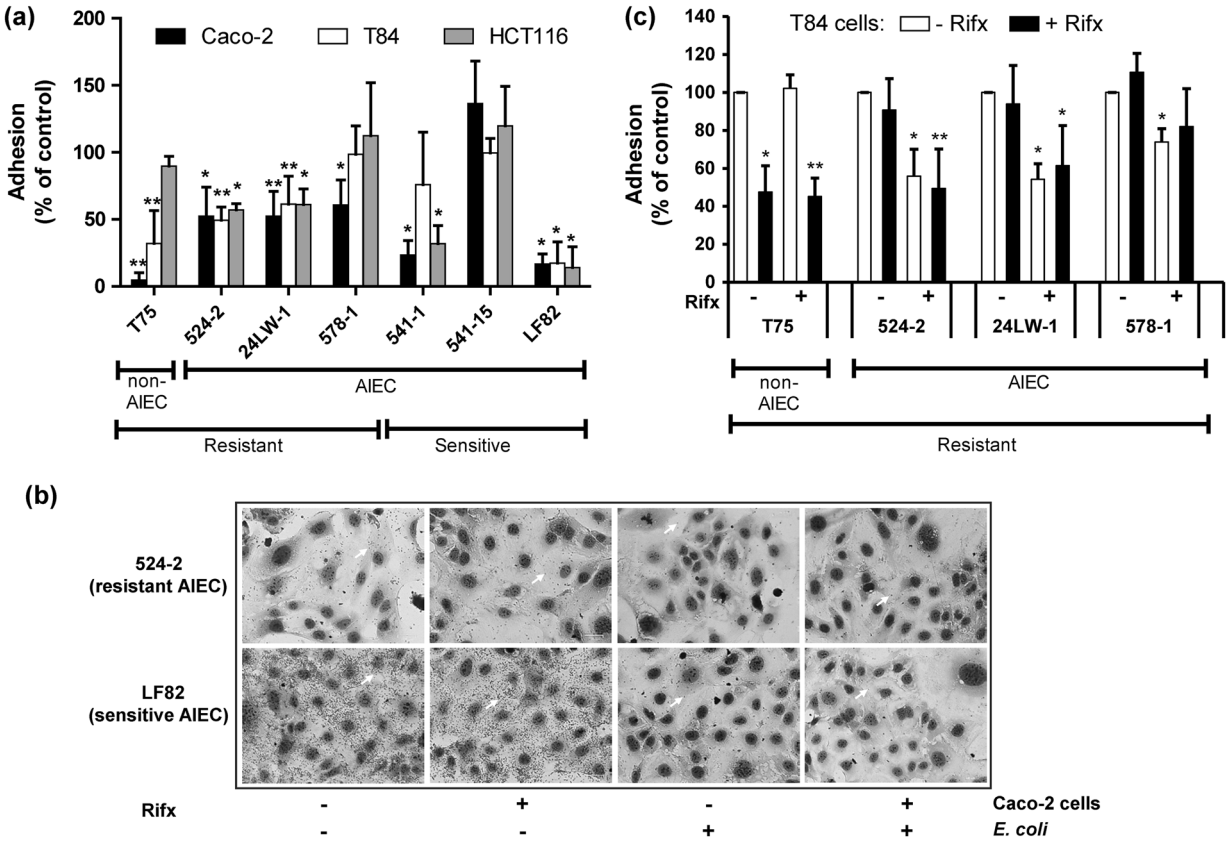

Fig. 1 Effect of rifaximin on adhesion of *E. coli* to intestinal epithelial cell lines. **a** Adhesion to Caco-2, HCT116, and T84 cells at 3 h post-infection. Both bacteria and epithelial cells were pre-treated with rifaximin, and rifaximin was kept in the cell culture media during infection. Control bacteria and cells were pre-treated with corresponding amounts of vehicle control (DMSO). Adhesion was calculated as the percentage of the adhered bacteria in the presence of rifaximin relative to the control. 100% indicates "no effect", less than 100% indicates decreased adhesion in the presence of rifaximin. Data are mean, error bars are SD. Data were analyzed for statistical significance using a two-tailed paired *t* test, $*p < 0.05$, $**p < 0.01$. **b** Giemsa stain of vehicle control or rifaximin-treated Caco-2 cells infected with rifaximin-resistant AIEC 524-2 and rifaximin-sensitive AIEC LF82. −, + signs indicates rifaximin pre-treatment of bacteria and/or Caco-2 cells. The arrow shows adherent bacteria. **c** The decrease in adhesion was due to a direct effect of rifaximin on bacteria in rifaximin-resistant AIEC *E. coli*. T84 epithelial cells and/or *E. coli* strains were pre-treated with rifaximin as indicated by − and + signs. Data are mean, error bars are SD. Data were analyzed for statistical significance using one-way ANOVA with Dunnett's multiple comparisons test (compared with control), $*p < 0.05$, $**p < 0.01$

Caco-2, T84, and HCT116 cells were infected with *E. coli* in the presence or absence of rifaximin as described above for the invasion assay. After 1 h the gentamicin concentration was reduced to 20 μg/ml to limit gentamicin diffusion into epithelial cells. After a total of 24 h, cell culture media was collected and analyzed by ELISA according to the manufacturer's specifications.

The production of TNFα by J774A.1 macrophages in response to *E. coli* infection ± rifaximin, was determined after 24 h of infection by ELISA (Invitrogen) according to the manufacturer's specifications.

Statistical analysis

Data were analyzed by paired *t*-test, one-way ANOVA with Dunnett's multiple comparisons test or two-way ANOVA with Bonferroni post-test where appropriate. All statistical analyses were performed with GraphPad Prism software, version 5.0 and $p < 0.05$ was considered significant.

Results

Rifaximin has a dose-dependent effect on the growth of sensitive, but not resistant, AIEC

Rifaximin inhibited the growth of sensitive AIEC in a dose-dependent manner (Supplementary Fig. S2a–c) with complete inhibition at 32 μg/ml. Sensitive strains were able to grow at 8 μg/ml (the rifaximin concentration used for sensitive strains in subsequent experiments). Rifaximin at concentrations up to 512 μg/ml had no effect on the growth of resistant strains (Supplementary Fig. S2d). We could not evaluate concentrations

> 512 µg/ml, because a precipitate formed at these concentrations.

Rifaximin decreases the adhesion of 5/6 AIEC to at least one intestinal epithelial cell line

The effect of rifaximin on the adhesion of AIEC to epithelial cells was determined using Caco-2, T84, and HCT116 epithelial cells. The absolute level of AIEC adhesion varied by strain and/or cell line (Supplementary Fig. S3). Our results illustrates that rifaximin decreases the adhesion of 5/6 AIEC to at least one intestinal epithelial cell line. Rifaximin decreased the adhesion of 3/3 rifaximin-resistant AIEC and non-AIEC T75 to Caco-2 epithelial cells (Fig. 1a, b). Adhesion to T84 and HCT116 cells was decreased in 2/3 rifaximin-resistant AIEC (Fig. 1a).

To determine if the decreased adhesion is due to the effect of rifaximin on bacteria or cells, we conducted additional cell culture experiments. T84 epithelial cells and/or *E. coli* strains were pre-treated with rifaximin. The decrease in adhesion was due to the effect of rifaximin on resistant AIEC (Fig. 1c) as treating T84 epithelial cells alone with rifaximin had no effect on adhesion, while treating bacteria regardless of the cell treatment reduced the bacterial adhesion. For one non-AIEC strain (T75) the effect was on T84 cells rather than bacteria (Fig. 1c). To determine if these findings were cell line specific, we repeated the experiment with 3 strains and HCT116 cells. The results for HCT116 paralleled those for T84 (Supplementary Fig. S4).

Rifaximin (at sub inhibitory concentrations) reduced the adhesion of sensitive AIEC, 541-1, and LF82, but not 541-15 (Fig. 1a, b). Among rifaximin-sensitive AIEC, the adhesion of 541-15 and LF82 was reduced at 32 µg/ml rifaximin, and was due to an effect on bacteria rather than epithelial cells (Supplementary Fig. S5a).

Rifaximin decreases invasion of intestinal epithelial cells by AIEC

Rifaximin significantly decreased the invasion of Caco-2, T84, and HCT116 epithelial cells by all rifaximin-resistant and rifaximin-sensitive AIEC and non-AIEC T75 (Fig. 2a). Because the invasion of Caco-2 cells by non AIEC T75 is very low, we excluded T75 from the Caco-2 invasion assay. The decrease in invasion of rifaximin-resistant strains was due to a direct effect of rifaximin on T84 epithelial cells rather than bacteria in all 4 resistant strains (Fig. 2b), since treating bacteria alone with rifaximin had no effect on invasion, while treating T84 epithelial cells with rifaximin reduced the bacterial invasion.

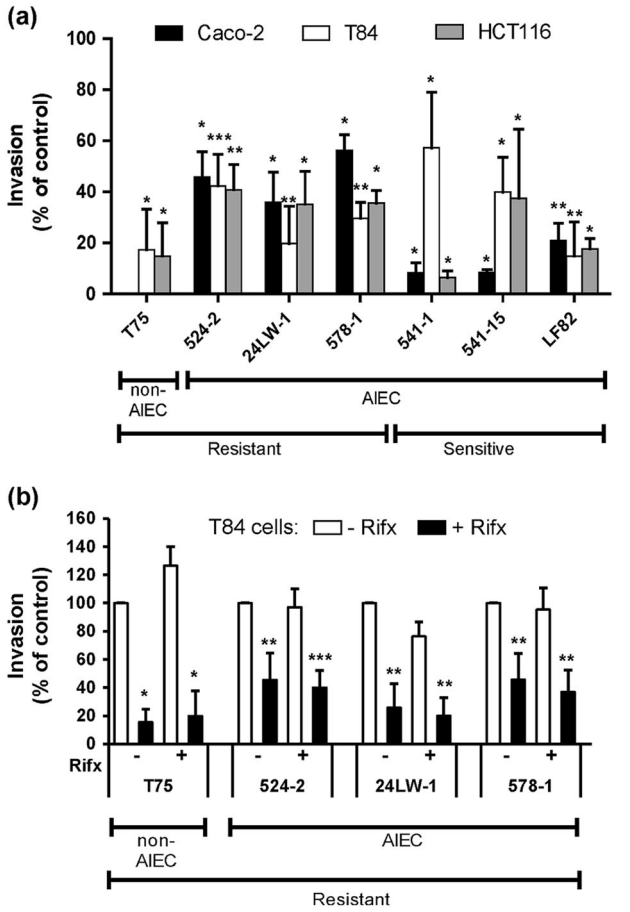

Fig. 2 Effect of rifaximin on invasion of intestinal epithelial cell lines by *E. coli*. **a** Intracellular bacteria in Caco-2, HCT116, and T84 cells after a 3 h infection period, followed by gentamicin treatment for 1 h. Both bacteria and epithelial cells were pre-treated with rifaximin. Rifaximin was kept in the cell culture media during infection. Control bacteria and cells were pre-treated with corresponding amounts of vehicle control (DMSO). Invasion was calculated as the percentage of the invasive bacteria in the presence of rifaximin relative to the vehicle control. 100% indicates "no effect", less than 100% indicates decreased invasion in the presence of rifaximin. Because the invasion of Caco-2 cells by non AIEC T75 is very low, we excluded T75 from the Caco-2 invasion assay. Data are mean, error bars are SD. Data were analyzed for statistical significance using a two-tailed paired *t* test, $*p < 0.05$, $**p < 0.01$. **b** The decrease in invasion was due to a direct effect of rifaximin on epithelial cells. T84 epithelial cells and/or *E. coli* strains were pre-treated with rifaximin as indicated by − and + signs. Data are mean, error bars are SD. Data were analyzed for statistical significance using one-way ANOVA with Dunnett's multiple comparisons test (compared with control), $*p < 0.05$, $**p < 0.01$

Among rifaximin-sensitive AIEC the decrease in invasion of LF82 was due to an effect on bacteria rather than epithelial cells. The decrease in invasion of 541-15 was due to the effect on both bacteria and cells and was concentration-dependent, as we did not see any change in invasion with 8 µg/ml rifaximin vs. 32 µg/ml (Supplementary Table S5b).

Table 2 Effect of rifaximin on the transcription of virulence genes in rifaximin-resistant and rifaximin-sensitive *E. coli*

Gene	Product	Function	Transcription (Relative quantity ± rifaximin, $2^{-\Delta\Delta Ct}$ [a])						
			Rifaximin-resistant *E. coli*				Rifaximin-sensitive *E. coli*		
			524-2	24LW-1	578-1	T75	541-15	541-1	LF82
fliC	Flagellin	Motility	**0.22*****	**0.46*****	**0.21*****	**0.22*****	**0.02*****	**0.01*****	**0.1*****
fimH	Type 1 fimbrial subunit	Adhesion	**0.33*****	**0.46*****	**0.20*****	**0.23*****	4.33***	**0.35*****	0.84
ompC	Outer membrane protein C	Outer membrane protein	**0.35***	**0.26*****	**0.30*****	**0.13*****	0.58	**0.10*****	0.77
yfgL	Lipoprotein	Invasion	**0.32****	**0.41*****	**0.51***	**0.53****	2.85**	0.73	2.47*
nlpI	Lipoprotein	Adhesion and Invasion	**0.27*****	**0.55****	**0.55***	**0.29*****	4.49***	0.54	1.26
$lpfA_{141}$	Long polar fimbrial protein A	M cell translocation	NA	NA	NA	NA	NA	NA	1.58
$lpfA_{154}$	Long polar fimbrial protein A	M cell translocation	0.76	NA	**0.33*****	NA	3.00**	0.99	NA
htrA	Periplasmic protease	Stress protein (Survival in macrophages)	**0.24*****	**0.46*****	**0.55***	**0.26*****	1.57	0.57	0.86
dsbA	Disulfide oxidoreductase	Oxidoreductase (Survival in macrophages)	0.70	1.19	**0.25*****	**0.60***	2.44*	1.37	1.44
fyuA	Ferric Yersiniabactin uptake receptor	Iron acquisition	NA	NA	**0.36*****	NA	2.27*	NA	1.84
chuA	Outer membrane heme/hemoglobin receptor	Heme iron acquisition	NA	NA	**0.05*****	NA	NA	NA	**0.39***

E. coli strains were grown to mid-log phase in LB broth in the presence of rifaximin (resistant strains: 128 µg/ml; sensitive strains: 8 µg/ml) or DMSO control

A value of >1 indicates upregulation and 1 indicates downregulation in the presence of rifaximin. Gene downregulation in the presence of rifaximin is indicated in bold

Statistical analysis was performed by two-way ANOVA. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$

NA gene is not present

[a] The average cycle threshold (Ct) value from each sample in triplicate was obtained for both target and housekeeping (*mdH*) genes. The relative quantity (RQ) was calculated by using the equation $RQ = 2^{-\Delta\Delta Ct}$

Rifaximin decreases the expression of virulence genes in rifaximin-resistant AIEC

Rifaximin decreased the expression of genes associated with AIEC motility, adhesion to, and invasion of epithelial cells, M cell translocation, iron acquisition, and survival in macrophage in rifaximin-resistant AIEC and non-AIEC (Table 2).

In rifaximin-sensitive strains the effect of sub-inhibitory concentrations of rifaximin was gene-specific and strain-specific (Table 2). Expression of *fliC* was significantly decreased in all rifaximin-sensitive AIEC. Type 1 pili adhesin *fimH* was upregulated in 541-15, downregulated in 541-1 and did not change in LF82.

To determine if rifaximin dose impacts virulence gene expression (*fliC*, *fimH*, *ompC*), we evaluated low dose rifaximin (8 µg/ml) in rifaximin-resistant strains. Our data indicate a dose-dependent effect, with less inhibition at eight than 128 µg/ml. However, 8 µg/ml rifaximin inhibited expression of *fliC* in all three resistant AIEC, but not in non-AIEC T75. In contrast, expression of *fimH* and *ompC* was only inhibited in one and two of four resistant strains, respectively (Supplementary Table S2). Therefore, some of the inhibitory actions of rifaximin are clearly dose-dependent. Because rifaximin concentration in feces can reach 7961 µg/g after 800 mg/day rifaximin treatment for 3 days, this loss of inhibition at concentrations less than 128 µg/ml may not be relevant in the clinical situation [26].

Rifaximin decreases the motility of AIEC

Rifaximin (128 µg/ml) decreased the motility of 3/3 rifaximin-resistant AIEC (Fig. 3a, b). The effect of rifaximin on the motility of rifaximin-sensitive AIEC was even more profound, with motility completely inhibited in the presence of 8 µg/ml rifaximin (Fig. 3a, b). These findings have parallel changes in *fliC* expression (Table 2). Non-AIEC T75 was not evaluated as it is non-motile.

Rifaximin reduces AIEC induced IL-8 secretion by T84 intestinal epithelial cells

Rifaximin decreased the secretion of IL-8 by T84 cells, but not Caco-2 and HCT116 cells infected with rifaximin-resistant AIEC (Fig. 4a). However, rifaximin treatment

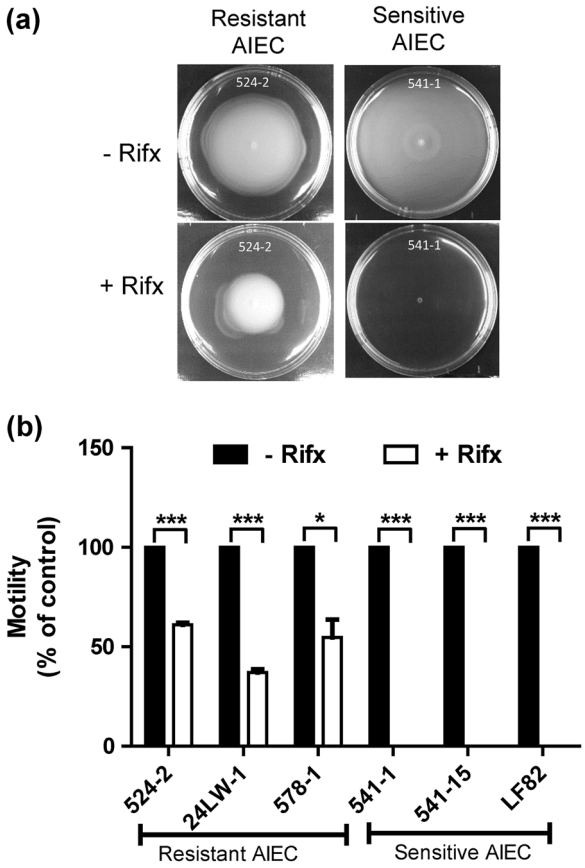

Fig. 3 Effect of rifaximin on the motility of AIEC. **a** Swarming motility on soft agar from the point of inoculation ± rifaximin. Rifaximin concentrations in agar were 128 µg/ml for resistant strains and 8 µg/ml for sensitive strains. **b** Inhibition of motility by rifaximin. Motility was calculated as the percentage of the swarm diameter on rifaximin positive relative to rifaximin negative plates. Data are mean, error bars are SD. *P* values were calculated by using a two-tailed paired *t* test, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

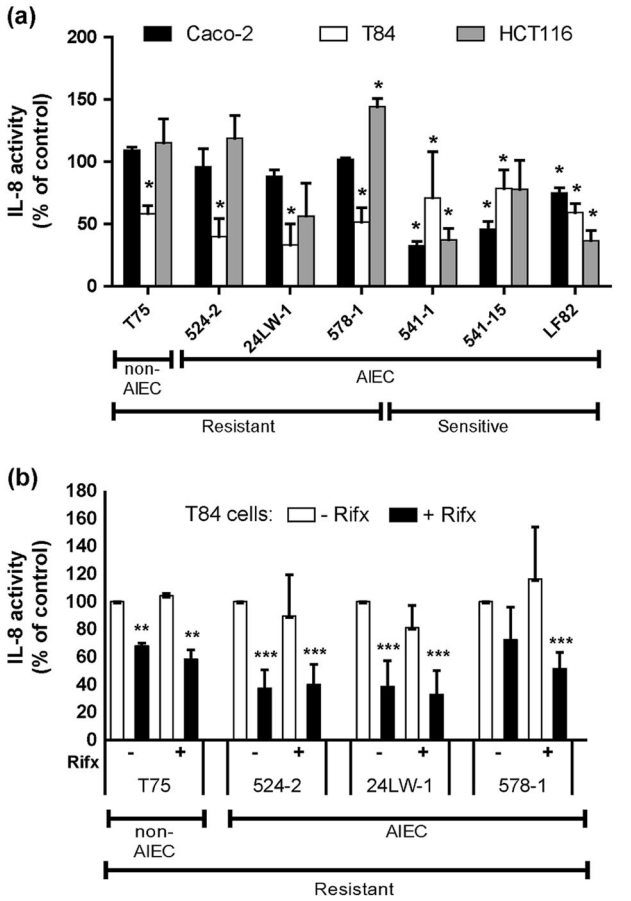

Fig. 4 Rifaximin reduces AIEC induced IL-8 secretion by T84 intestinal epithelial cells. **a** Effect of rifaximin on IL-8 secretion by Caco-2, T84, and HCT116 cells in response to *E. coli*. Both bacteria and epithelial cells were pre-treated with rifaximin, and rifaximin was kept in the cell culture media during infection. Control bacteria and cells were pre-treated with corresponding amounts of vehicle control (DMSO). Cells were infected with *E. coli* for 3 h and extracellular bacteria were killed with gentamicin for 1 h. After a total of 24 h cell culture media was collected and analyzed by ELISA for IL-8. IL-8 activity was calculated as the percentage of the IL-8 in the presence of rifaximin relative to the control. 100% indicates "no effect", less than 100% indicates decreased IL-8 production in the presence of rifaximin. Data are mean, error bars are SD. Data were analyzed for statistical significance using a two-tailed paired *t* test, $*p < 0.05$. **b** The decrease in IL-8 expression was due to a direct effect of rifaximin on epithelial cells. T84 epithelial cells and/or *E. coli* strains were pre-treated with rifaximin as indicated by − and + signs. Data are mean, error bars are SD. Data were analyzed for statistical significance using one-way ANOVA with Dunnett's multiple comparisons test (compared with control), $*p < 0.05$, $**p < 0.01$

reduced IL-8 secretion from all three cell lines infected with rifaximin-sensitive AIEC (Fig. 4a).

The effect of rifaximin on IL-8 secretion in response to rifaximin-resistant AIEC was due to a direct effect on T84 cells (Fig. 4b). The decrease of IL-8 secretion in response to rifaximin-sensitive AIEC was due to the effect on only bacteria in LF82, and also on both bacteria and T84 cells in 541-1 and 541-15, but this appears to be concentration-dependent, as we did not see any change in IL-8 secretion when we treated the epithelial cells with 8 µg/ml rifaximin as opposed to 32 µg/ml (Supplementary Fig. S5c).

We also evaluated the effect of rifaximin on IL-6 and IL12p40 secretion by Caco-2, T84, and HCT116 cell lines. There was no detectable IL-6 and IL12p40 in response to *E. coli* infection in the absence or presence of rifaximin (data not shown).

Rifaximin decreases survival of E. coli in macrophages

Rifaximin decreased the ability of 1/3 resistant and 2/3 sensitive AIEC to replicate and survive within macrophages (Supplementary Fig. S6a). The most profound effect was observed with prototypical AIEC LF82 where survival

was 1% of the control. Non-AIEC T75 does not replicate in macrophages but rifaximin enhanced the killing of T75 in J774 macrophages.

TNF production by J774 macrophages in response to infection with rifaximin-resistant AIEC was higher in the presence of rifaximin (Supplementary Fig. S6b). Conversely, TNF production by macrophages in response to infection with 2/3 sensitive AIEC was inhibited by rifaximin.

Discussion

The consistent association of dysbiosis and selective enrichment of AIEC in CD suggests the potential therapeutic benefit of selectively targeting disease-associated pathosymbionts with antibiotics. This is supported by the ability of rifaximin, a non-absorbable antibiotic developed to target *E. coli* associated with travelers' diarrhea [11], to induce remission in CD patients [12, 27]. However, it is unclear if this outcome is due to its effect on bacteria or intestinal epithelial inflammatory responses. The isolation of rifaximin-resistant *E. coli* from the ileum of CD patients treated with rifaximin indicates that antimicrobial resistance could influence outcome [14, 28]. We have shown that rifaximin is able to reduce virulence gene expression, motility, and adhesion of the majority of CD-associated AIEC in a manner independent of its antimicrobial activity. The effect of rifaximin on invasion and cytokine secretion by PXR expressing T84 cells was largely attributable to its action on epithelial cells rather than bacteria.

Adhesion to and invasion of epithelial cells is central to the AIEC pathotype. Our findings indicate that rifaximin is able to reduce the adhesion to and invasion of CD-associated AIEC in three different epithelial cell lines. We found that the effect of rifaximin on adhesion was attributable to its effect on bacteria and was independent of its antimicrobial activity. The reduced ability of AIEC to adhere to epithelial cells may reflect decreased motility due to its effect on flagella (FliC). Flagella-mediated motility plays an important role for AIEC at various stages of the infection and flagella-mediated movement in the intestinal lumen is required for efficient colonization and induction of colitis in a murine gastroenteritis model [10, 29]. Our findings could also be explained by decreased expression of FimH of type I pili in AIEC, considered a major determinant of adhesion to epithelial cells via recognition of oligomannosides on carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) [30] and TLR4. Additionally, reduced expression of *nlpI*, *yfgL*, and *ompC* in rifaximin-resistant AIEC could also decrease adhesion. Deletion of *nlpI* in AIEC strain LF82 decreases adhesion and expression of type 1 pili and flagella [21]. *yfgL* expression has been correlated with outer membrane vesicle secretion (ompC) which impacts synthesis of flagellae and type I pili through the RpoE regulatory pathway [24, 25]. While rifaximin reduced expression of *fliC, fimH, nlpI, yfgL*, and *ompC*, how or where rifaximin impacts this group of genes remains to be determined.

A previous study found that pretreating laryngeal epithelial cells (HEp-2), epithelial cells of lung (A549), and cervical cancer cells (HeLa) with rifaximin significantly decreased the adhesion of enteroaggregative *E. coli* (EAEC) [20]. However, unlike this prior study, we did not observe an effect of rifaximin pretreatment of three intestinal epithelial cells lines on the adhesion of AIEC.

In contrast to the antibacterial effect of rifaximin on adhesion, its effects on invasion were predominantly on the epithelial cell. This was somewhat unexpected given that the effect of rifaximin on bacterial motility and expression of virulence factors associated with adhesion and invasion (*fimH, nlpI, yfgL, ompC*). Previous studies suggest that rifaximin can reduce bacterial invasion by changing epithelial cell physiology [20]. Pretreating lung epithelial cells (A549) with rifaximin reduced invasion by *Bacillus anthracis* but not *Shigella sonnei*, which utilize different invasion mechanisms. Protein expression profiling of HEp-2 incubated with rifaximin has revealed changes in the expression of cytoskeletal proteins [31]. Invasion of cultured epithelial cells by AIEC is associated with cytoskeletal rearrangement and is inhibited by disruption of microtubules and microfilaments by colchicine and cytochalasin, respectively [32]. Whether the rifaximin induced alterations in the epithelial cytoskeletal account for the reduced invasion of AIEC remains to be determined.

The production of cytokines by intestinal epithelial cells is crucial for coordinating effective immune responses against invasive pathogens. In this study we demonstrated that rifaximin reduces IL-8 secretion by *E. coli* infected T84 cells. IL-8 is a cytokine precursor of polymorphonuclear leukocyte inflammatory response that is overexpressed in CD patients, and induced by AIEC [33]. Our results suggest that reduced IL-8 expression is due to the effect of rifaximin on the epithelium and not the bacterium. Rifaximin is a gut-specific agonist of the human pregnane X receptor (PXR) [34], which is a nuclear receptor and transcription factor that modulates drug transport and also regulate the inflammatory response [35]. Reduced IL-8 secretion from T84 cells, but not Caco-2 or HCT116, is consistent with rifaximin-induced activation of PXR [36], which inhibits pro-inflammatory transcription factor NF-κB, and down-regulates of NF-κB regulated genes [35]. The differential effect of rifaximin on IL-8 secretion by Caco-2 and HCT116 cells correlates with the relative abundance of PXR in these cell lines relative to T84 [37, 38]. Low levels of PXR activity has been associated with IBD [36, 39] and activation of PXR by rifaximin

has been reported to inhibit pro-inflammatory transcription factor NF-κB, and down-regulation of NF-kB target genes expression [34, 35]. The ability of rifaximin to inhibit AIEC induced IL-8 expression by PXR expressing colonic epithelial cells suggests it may have a similar mechanism in patients with IBD.

An important trait of AIEC is their ability to survive and replicate in macrophages [40]. Rifaximin decreased the survival of one of the three resistant AIEC strains. The ability of AIEC to survive in macrophages involves the protease HtrA and the thiol-disulfide oxidoreductase DsbA [22, 23]. It is notable that rifaximin treatment decreased *htrA* expression in all and *dsbA* expression in two of the three rifaximin-resistant AIEC strains. Survival of AIEC in macrophages also depends on TNF-alpha secretion, which is considered to stimulate the proliferation and survival of AIEC [41]. While *htrA* and *dsbA* expression was reduced in rifaximin-resistant AIEC, the production of TNF-alpha by infected macrophages increased. This increase in TNF-alpha concentrations may have counteracted the effect of reduced *htrA* and *dsbA* expression on AIEC survival in our study. In sensitive AIEC, rifaximin decreased survival and replication of two of the three strains but did not reduce *htrA* and *dsbA* expression, and decreased TNF-alpha secretion. Thus, the effect of rifaximin on survival and replication of AIEC within macrophages is strain and pathotype dependent and remains to be fully elucidated.

In conclusion, we have found that rifaximin has a multifaceted impact on the AIEC pathotype. The use of *E. coli* strains which are resistant to rifaximin mediated killing and impairment of growth enabled us to study the effects of rifaximin that are independent of its ability to kill bacteria. Our results showed that rifaximin interferes with multiple steps implicated in host-AIEC interactions related to CD, including virulence gene expression, motility, adhesion and invasion of epithelial cells, persistence in macrophages, and pro-inflammatory cytokine secretion. Because CD is a complex disease, with strong evidence of interplay between the enteric microflora e.g., AIEC, the intestinal mucosa, and immune effector cells, the pluripotent activity of rifaximin make it an appealing candidate for the treatment of AIEC associated intestinal inflammation. Further study is required to determine the relationship of these in vitro effects to clinical responses in patients with CD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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