

ORIGINAL ARTICLE

The human milk oligosaccharide 2'-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation

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► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/gutjnl-2014-307544>).

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Received 6 May 2014
Revised 29 October 2014
Accepted 31 October 2014
Published Online First
27 November 2014



CrossMark

To cite: He YY, Liu SB, Kling DE, et al. Gut 2016;**65**:33–46.

ABSTRACT

Background A major cause of enteric infection, Gram-negative pathogenic bacteria activate mucosal inflammation through lipopolysaccharide (LPS) binding to intestinal toll-like receptor 4 (TLR4). Breast feeding lowers risk of disease, and human milk modulates inflammation.

Objective This study tested whether human milk oligosaccharides (HMOs) influence pathogenic *Escherichia coli*-induced interleukin (IL)-8 release by intestinal epithelial cells (IECs), identified specific proinflammatory signalling molecules modulated by HMOs, specified the active HMOs and determined its mechanism of action.

Methods Models of inflammation were IECs invaded by type 1 pili enterotoxigenic *E. coli* (ETEC) in vitro: T84 modelled mature, and H4 modelled immature IECs. LPS-induced signalling molecules co-varying with IL-8 release in the presence or absence of HMOs were identified. Knockdown and overexpression verified signalling mediators. The oligosaccharide responsible for altered signalling was identified.

Results HMOs attenuated LPS-dependent induction of IL-8 caused by ETEC, uropathogenic *E. coli*, and adherent-invasive *E. coli* (AIEC) infection, and suppressed CD14 transcription and translation. CD14 knockdown recapitulated HMO-induced attenuation. Overexpression of CD14 increased the inflammatory response to ETEC and sensitivity to inhibition by HMOs. 2'-fucosyllactose (2'-FL), at milk concentrations, displayed equivalent ability as total HMOs to suppress CD14 expression, and protected AIEC-infected mice.

Conclusions HMOs and 2'-FL directly inhibit LPS-mediated inflammation during ETEC invasion of T84 and H4 IECs through attenuation of CD14 induction. CD14 expression mediates LPS-TLR4 stimulation of portions of the 'macrophage migration inhibitory factors' inflammatory pathway via suppressors of cytokine signalling 2/signal transducer and activator of transcription 3/NF- κ B. HMOs direct inhibition of inflammation supports its functioning as an innate immune system whereby the mother protects her vulnerable neonate through her milk. 2'-FL, a principal HMOs, quenches inflammatory signalling.

INTRODUCTION

Breastfed infants have lower risk of inflammatory conditions than those fed artificially.¹ Human milk suppresses inflammatory processes in vitro, and

Significance of this study

What is already known on this subject?

- Major causes of enteric infection, Gram-negative bacteria, activate mucosal inflammation through lipopolysaccharide (LPS) binding to intestinal toll-like receptor 4 (TLR4).
- Breast feeding lowers risk of disease, and human milk modulates inflammation.
- Oligosaccharides are collectively the third largest components of human milk.

What are the new findings?

- Human milk oligosaccharides (HMOs) attenuate LPS-dependent induction of interleukin (IL)-8 caused by enterotoxigenic *Escherichia coli*, uropathogenic *E. coli* and adherent-invasive *E. coli* infection.
- CD14 levels mediate LPS-dependent IL-8 inflammatory response.
- HMOs suppress CD14 mRNA transcription and CD14 translation and trafficking.
- 2'-fucosyllactose (2'-FL) upregulates suppressors of cytokine signalling 2 and phosphorylation of signal transducer and activator of transcription 3, and suppresses CD14 expression and release of IL-8, accounting for total HMOs activity in these infection models.

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

- 2'-FL directly attenuating inflammation further confirms HMOs as an innate immune system of human milk whereby the mother protects her vulnerable neonate; this supports universal breast feeding as a standard of care.
- 2'-FL may represent a novel oral prophylactic and therapeutic agent to quench mucosal inflammation associated with diverse inflammatory disorders of the mucosa.

anti-inflammatory components have been documented,² including the oligosaccharide fraction (human milk oligosaccharide, HMOs), a heterogeneous mixture of complex carbohydrate structures.³ HMOs are comprised of at least 200 individual oligosaccharides belonging to 12 different core structural groups with lactose at the

reducing end, and consist of glucose, galactose, N-acetyl-glucosamine, fucose and sialic acid.^{4–6} We hypothesise that HMOs constitute an innate immune system of human milk whereby breastfeeding mothers protect their infants from enteric pathogens through three distinct mechanisms: inhibiting pathogen adhesion, prebiotic enhancement of mutualist microbiota and immunomodulation.^{3 7}

Inhibition of pathogen adhesion emerges from structural homology between HMOs and mucosal cell surface glycans. Being soluble epithelial cell membrane receptor analogues, HMOs competitively inhibit binding of enteric pathogens to epithelial cell membranes.^{3 8} As functional prebiotics, HMOs promote the growth of beneficial mutualist bacteria, such as *Bifidobacterium bifidum*, thereby influencing the colonisation and ultimate composition of intestinal microbiota.^{5 9 10} HMOs preparations stimulate immunomodulatory activity on the neonatal intestinal mucosal surface^{9 11–13} and modulate cytokine production.^{14–18} The relative activities of individual oligosaccharides and their specific mechanisms of modulating individual pathways have not been defined.

Colostrum HMOs modulate toll-like receptor 3 (TLR3), TLR5 and interleukin 1 β (IL-1 β) dependent pathogen-associated molecular patterns (PAMPs) signalling pathways.¹⁸ Recognition of PAMPs by pattern recognition receptors (PRRs), including TLRs and nod-like receptors,^{19 20} is a primary mechanism by which intestinal epithelial cells (IECs) interact with the microbiota, including pathogens.^{21–23} When encountering microbe-specific ligands, PRRs initiate signal transduction pathways, which activate downstream nuclear transcription factors that modulate control elements of specific genes to induce transcription of proinflammatory cytokines and chemokines; their expression governs innate and adaptive responses to foreign microbes.^{19 20}

The outermost membrane of Gram-negative bacteria contains lipopolysaccharides (LPS), a glycolipid PAMP.²⁴ Pathogen-associated LPS strongly elicits several immediate proinflammatory responses in mammalian cells.^{25 26} The LPS receptor is a complex comprised of LPS-binding protein, MD2, CD14 and TLR4.²⁷ Thus, CD14 is essential for the LPS recognition and binding that induce inflammatory signaling cascades.²⁸ Generally, CD14 is anchored to the cell membrane by its glycosylphosphatidylinositol (GPI) moiety.²⁹ Upon LPS recognition, TLR4s oligomerise and recruit proteins containing TIR (toll-IL-1 receptor) domains, such as myeloid differentiation primary response gene 88 (MyD88),²⁵ promoting cascades that induce NF- κ B activation and translocation to the nucleus, ultimately inducing cytokine expression.³⁰ In most circumstances, this reaction to LPS is adaptive: During early colonisation, and during maintenance of mature microbiota, it is a component of mucosal barrier defence by restraining native microbiota to the lumen of the gut.³¹ This primary response to pathogen invasion thwarts pathogen passage into the body and helps clear invading microbial organisms.²⁵ Conversely, the induction of excessive inflammation by LPS is a major component of the pathogenesis of enteropathogen infection. For example, invasion by type 1 pili *Escherichia coli*, such as uropathogenic *E. coli* (UPEC) and enterotoxigenic *E. coli* (ETEC), induces LPS-dependent proinflammatory IL-8 secretion.³² An abnormal TLR4-dependent reaction to LPS may underlie the pathogenesis of IBD.³³

This study tested the hypothesis that HMOs suppress LPS-induced inflammation in IECs infected by type 1 pili *E. coli*, measured as attenuated IL-8 secretion in ETEC-infected T84 cells and adherent-invasive *E. coli* (AIEC)-infected mice.

The activities of individual oligosaccharides were assessed, and the mechanism of inhibition was identified through construction of a signalling pathway map.

MATERIALS AND METHODS

Isolation of HMOs

HMOs were prepared from pooled human milk,¹⁸ and endotoxin reduced to 0.01 LAL units (Genscript USA, Piscataway, New Jersey, USA) by a polymyxin B column. Pure 2'-fucosyllactose (2'-FL), 3-FL and lacto-N-fucopentaose I (LNFP I) were from Glycosyn (Waltham, MA). 3'-sialyllactose (3'-SL) and 6'-SL were from Carbosynth (Compton, UK). Trifucosyl (1,2-1,2-1,3)-iso-lacto-N-octose (TFILNO) was from Glucose separations (Moscow, Russia).

Cell lines and bacteria

IEC strains (T84/HCT8/FHs74) and HeLa cells were from ATCC (Manassas, Virginia, USA). T84 and HeLa cells were cultured in DMEM/F12 medium (Invitrogen, Grand Island, New York, USA) containing 10% FBS (Atlas Biologicals, Fort Collins, Colorado, USA). H4 cells from Dr Lei Lu (Massachusetts General Hospital), were cultured in DMEM/F12 containing 10% FBS and 30 ng/mL EGF. HCT8 was cultured in RPMI 1640 (Sigma, St. Louis, Missouri, USA) containing 10% FBS. FHs74 was cultured in X-46 medium (ATCC) containing 10% FBS and 30 ng/mL EGF. All cells were cultured at 37°C in a water-saturated atmosphere of 5% CO₂.

ETEC (H10407) was purchased from ATCC. AIEC (LF82) was from Dr Jakob Møller-Jensen (University of Southern Denmark). UPEC (CF073) was from Dr Harry Mobley (University of Michigan Medical School). *Salmonella enterica* serovar Typhimurium (SL1344) was provided by Dr Bobby J Cherayil (Massachusetts General Hospital and Harvard). Bacteria were cultured at 37°C in Difco nutrient medium (BD Biosciences, San Jose, California, USA) at 225 rpm.

LPS stimulation in vitro

IECs were cultured at 5×10^4 cells per well (subconfluent) in 24-well plates (Corning Life Sciences, Tewksbury, Massachusetts, USA) for 48 h in 500 μ L media containing HMOs approximating physiological levels (HMOs, 5 mg/mL; 2'-FL, 2 mg/mL; 3-FL, 0.4 mg/mL; 6'-SL, 0.5 mg/mL; 3'-SL, 0.5 mg/mL; LNFP I, 2.5 mg/mL; TFILNO, 3 μ g/mL), followed by LPS (*E. coli*, Sigma, St. Louis, Missouri, USA) stimulation (T84 cells 100 μ g/mL; H4 cells 200 ng/mL) for 16 h. Supernatants were stored at -20°C until analysis.

ETEC stimulation in vitro

IECs were seeded at 5×10^4 cells per well and cultured for 48 h in 500 μ L of fresh media containing 5 mg/mL HMOs. Cells were washed with PBS twice and provided with fresh antibiotic-free, milk oligosaccharide-free media. IECs were inoculated with suspensions of ETEC with a multiplicity of infection (MOI) of 20, and incubated for 1 h. From these, the number of invading bacteria (i), the number of adherent bacteria (a) and the inflammatory response of the IECs were measured as described in online supplementary material.^{34–36}

Inhibition of ETEC infection by cytochalasin D

Cytochalasin D, an inhibitor of actin polymerisation, inactivates host cell machinery needed for invasion by bacteria. Cytochalasin D (2 μ M) was added to media of T84 cells for 30 min before ETEC inoculation (MOI=20) to inhibit invasion,

and after exposure to ETEC, the ability of 2'-FL to inhibit IL-8 expression was measured in six replicate experiments.

IL-8 ELISA

The inflammatory response of T84 or H4 IECs after LPS stimulation or bacterial invasion was measured as IL-8 by ELISA (R&D Systems, Minneapolis, Minnesota, USA) in defrosted supernatants that had been centrifuged for 5 min at 4°C and 15 700×g. The colour generated at 450 nm was measured on a Versa Max plate reader (Molecular Devices, Sunnyvale, California, USA). Values were normalised to cell number (by alamarBlue).

Quantitative RT-PCR

T84 cells treated with milk oligosaccharides were stimulated with LPS or ETEC, and simultaneously analysed for CD14 protein and mRNA, as described in online supplementary material, to increase the concordance between these two types of data.

Flow cytometry analysis

Cell suspensions from T84 cells (5×10^5 cells/well) treated with or without HMOs were incubated with PE-Cy7 labelled mouse antihuman CD14 mAb (BD Biosciences, San Diego, California, USA) on ice for 30 min. PE-Cy7 conjugated mouse IgGk isotype-matched (IgG1) antibodies (BD Biosciences, San Diego, California, USA) were controls. Fluorescence was by FACScan (BD Biosciences, San Diego, California, USA), 20 000 live cells per data point, and data analysed by Accuri C6 flow cytometer software (BD Biosciences, San Diego, California, USA).

Western blots

Western blot analyses were performed as described in online supplementary material.

CD14 knockdown in IECs

To knock down CD14 translation, CD14 shRNA was transfected into T84 cells using a pRS lentiviral expression vector (OriGene Technologies, Rockville, Maryland, USA). Negative controls were vector only and vector with scrambled shRNA. Suppression of CD14 was assessed by western blot analysis 72 h post plasmid transfection.

Overexpression CD14 in HeLa cells

Overexpression of CD14 is toxic to T84, HCT8, FHs74 and H4 human IEC lines, but not to HeLa cells, whose intrinsic expression of CD14 is low. HeLa cells (2.5×10^6) were transfected with the pCDNA3-huCD14 plasmid by Lipofectamine 2000 for 24 h. pCDNA3 blank vector (1 µg) was a control. Overexpression of CD14 was assessed by western blot 48 h after transfection, and expressed as fold induction relative to β-actin.

Antibody arrays

T84 cells were treated with 2'-FL. Proteins were extracted, labelled, hybridised to antibody array slides and scanned. Data were analysed by ScanArrayGx/ProScanarray software, and proteins whose expression was significantly altered by the 2'-FL treatment were clustered according to signalling regulation networks by Metacore software as described in online supplementary material.

In vivo study

A murine model of AIEC infection.^{37 38} was adapted and validated. Eight-week-old female C57BL/6 mice (Charles River Laboratories) received 0.25% dextran sodium sulfate (DSS) in their drinking water for 3 days, and were given 20 mg of streptomycin by gavage on day 4; half also received 100 mg of 2'-FL in 200 µL by gavage for each of the 4 days. On the 5th day, the two groups of experimental mice were inoculated with 10^9 colony forming unit (CFU) AIEC via 200 µL gavage and sacrificed after an additional 4 days; a control group received DSS and antibiotic, but only a sham PBS inoculation. Body weight was monitored daily. AIEC in faeces and colonic tissue were quantified as CFU on erythromycin/ampicillin LB plates.³⁷ Formalin (4%) fixed, paraffin-embedded 5 µm sections of mouse colon were stained with H&E. Cryosections (5 µm) of mouse colons stained with CD14 or O83 antibodies were studied by confocal microscopy. Total RNA was extracted from other colonic samples with Trizol for real-time quantitative PCR of CD14 mRNA levels, and protein was extracted for ELISA analysis of proinflammatory cytokines.

Statistical analysis

Data are presented as mean ± SEM; the significance of differences was determined by post hoc analysis of variance with Bonferroni correction, (Prism software; GraphPad Software, San Diego, California, USA). Array data was analysed by GenePix Pro software. p Values of 0.05 or less are considered statistically significant. Western blot figures and photomicrographs are representative of at least three concordant independent experiments.

RESULTS

HMOs inhibit ETEC invasion and IL-8 secretion

Cells pretreated with HMOs displayed reduced ETEC adherence and invasion (see online supplementary figure S1A,B), and IL-8 release (see online supplementary figure S1C) in a time-dependent manner (see online supplementary figure S1D). Higher concentrations could not compensate for shorter incubation (not shown).

HMOs suppress CD14 expression

Of CD14, TLR4, MyD88, NF-κB and IL-8, the most pronounced attenuation by HMOs was CD14 mRNA (see online supplementary figure S2A) and CD14 protein expression (see online supplementary figure S2B, western blot). Cell-surface CD14 was also reduced (see online supplementary figure S2C). Both processes were inhibited by HMOs irrespective of the elevated signalling during ETEC infection (see online supplementary figure S2A,B).

T84 cells exhibit high endogenous CD14 expression (see online supplementary figure S3A), that is knocked down by shRNA transfection (see online supplementary figure S3B), decreasing IL-8 induction (see online supplementary figure S3C). Knockdown of CD14 diminished the ability of HMOs to inhibit the IL-8 induction (see online supplementary figure S3C), demonstrating that HMOs attenuation of inflammation depends upon CD14 expression.

HeLa cells express low intrinsic levels of CD14 (see online supplementary figure S3A) whose overexpression is induced by transfection with pCDNA3-huCD14 plasmids (see online supplementary figure S4B). In untreated HeLa cells, increased IL-8 secretion caused by ETEC infection is presumably mediated by factors other than CD14, and HMOs did not inhibit this IL-8

induction (see online supplementary figure S4A). In contrast, overexpressing CD14 resulted in HMOs suppressing ETEC-induced IL-8 secretion ($p < 0.01$) (see online supplementary figure S4C). Accordingly, CD14 induction, the presumed target of HMOs, was used to differentiate activities of individual oligosaccharides.

2'-FL suppresses CD14 expression

LPS mediates proinflammatory signalling during UPEC invasion of bladder epithelium³² and ETEC invasion of T84 cells (not shown). LPS at 100 µg/mL triggered significant release of IL-8 from T84 cells (see online supplementary figure S5) and HMOs attenuated this induction (figure 1A). In this more facile simplified model, 2'-FL, 3-FL, 6'-SL, 3'-SL, LNFP I and TFILNO (figure 1B) were tested individually at their concentrations in human milk^{39–41} for ability to attenuate inflammation (figure 1C). Only 2'-FL suppressed LPS-induced induction of IL-8 levels in T84 cells (figure 1B). At 2 mg/mL, 2'-FL decreased the IL-8 secretion induced in LPS-treated T84 cells by 45%, similar to the 50% inhibition by 5 mg/mL HMOs (figure 1B). Inhibition by 2'-FL is dose-dependent (figure 1D, E) and plateaus at 80% inhibition of IL-8 induction by 4 mg/mL 2'-FL (figure 1E). Cytochalasin D inhibits the intracellular actin machinery in T84 cells that ETEC use for invasion. Incubation with 2 µM cytochalasin D did not affect basal expression of IL-8 in the presence or absence of 2'-FL (figure 1F, bars 5 and 6). Cytochalasin D pretreatment of T84 cells before ETEC infection reduced induction of IL-8 after exposure to ETEC (figure 1F, bars 3 and 7). The inability of 2'-FL to inhibit residual IL-8 expression (figure 1F, bars 7 and 8), indicates that 2'-FL inhibits only the IL-8 induced specifically by ETEC invasion.

At 2 mg/mL, the nominal concentration in most human milk, 2'-FL suppressed CD14 mRNA (figure 2A) and reduced cell-associated CD14 protein expression (figure 2B). Conversely, concentrations of soluble CD14 increased in the cell supernatants (figure 2C), and membrane-associated CD14 relocated to the cytoplasm (confocal microscopy) in 2'-FL-treated cells (figure 2D), for basal ($p < 0.05$) and LPS-treated cells ($p < 0.05$). Thus, 2'-FL, the major HMOs, accounts for the ability of HMOs to suppress cell-associated CD14 expression and to attenuate LPS stimulated IL-8 secretion in T84 cells.

While T84 cells are considered a model for enterocytes of mature intestinal mucosa, neonates consuming human milk have an immature gut more sensitive to inflammatory agents.⁴² The H4 enterocyte cell line models immature intestine, and is more sensitive to LPS than T84. In H4 cells, 200 ng/mL LPS induced significant release of IL-8 (figure 2E); HMOs mitigated these changes. Thus, in mature and immature IECs, 2'-FL, the major HMOs component, accounts for the ability of HMOs to attenuate LPS stimulated IL-8 secretion and suppress cell-associated CD14 expression.

2'-FL ameliorates inflammation induced by bacterial invasion

Consistent with the above effect on LPS, 2 mg/mL 2'-FL inhibited invasion of ETEC in T84 cells and suppressed the associated IL-8 induction (figure 3A) comparably with the 5 mg/mL HMOs seen in online supplementary figure S1. To ensure that the inhibition displayed by 2'-FL is not idiosyncratic to the T84 cell line, 2'-FL activity was tested in HCT8 cells, an IEC human ileal cell line that is especially sensitive to ETEC invasion. In these HCT8 cells, pretreatment for 48 h with 2'-FL at 2 mg/mL

inhibited ETEC invasion and attenuated the consequent IL-8 secretion (figure 3B).

To test whether the above observations were general to other type 1 pili organisms, the ability of 2'-FL to inhibit inflammatory signalling was tested in two additional type 1 pili *E. coli* AIEC (strain LF82) and UPEC (strain CF073). As a control, the activity of 2'-FL was tested on invasion of T84 by *Salmonella enterica* serovar Typhimurium (strain SL1344), whose invasion is independent of type 1 pili, instead requiring the type III secretion system or Zipper-like or Trigger-like entry processes.⁴³ 2'-FL inhibited AIEC and UPEC invasion of T84 cells by ~50% (figure 3C, D) and the IL-8 production by ~25% and ~40%, respectively (not shown). Although SL1344 invasion was inhibited by total HMOs, it was not inhibited by 2'-FL (figure 3E). This suggests that HMOs components other than 2'-FL may inhibit other mechanisms of pathogenesis, but type 1 pili pathogenesis is specifically inhibited by 2'-FL.

2'-FL induces macrophage migration inhibitory factor signal pathways that suppress inflammation

Changes in intracellular signalling associated with 2'-FL-induced changes in CD14 expression were studied in T84 cells. Signalling molecules were measured via an array of 512 antibodies to signalling proteins. Cy5/Cy3 fluorescent signal ratios were analysed using GenePix Pro array analysis software. Filtering criteria were set as internally normalised ratios of > 1.3 or < 0.77 based on statistical significance with correction for multiple comparisons.⁴⁴

By these criteria, 2'-FL treatment of cells significantly modulated 28 signalling molecules (table 1). Functional analysis of these microarray data was conducted using integrated software from Metacore (GeneGo, <http://trials.genego.com>). Subsets of the macrophage migration inhibitory factor (MIF) inflammatory signalling network exhibited 2'-FL-induced changes that matched the 2'-FL-induced anti-inflammatory outcomes (see online supplementary figure S6). These changes in antibody microarray ligands induced by 2'-FL treatment were confirmed by western blot of LPS/TLR4 signal pathway mediators: 2'-FL depressed expression of CD14 and NF-κB, while inducing expression of IκB, a negative regulator of the NF-κB signal pathway (figure 4A). TLR4 and MyD88 expression was not changed (figure 4A). Erk phosphorylation decreased while p38 and Akt phosphorylation increased (figure 4A). Among the suppressors of cytokine signalling (SOCSs), 2'-FL increased expression of SOCS2 but not SOCS1 or SOCS3 (figure 4A). 2'-FL increased the phosphorylation (activation) of signal transducer and activator of transcription 3 (STAT3), a downstream signalling molecule shared by several SOCS pathways, but not of STAT1 (figure 4A). Changes in western blot intensity for all measured signalling molecules are shown in figure 4B. In H4 cells, (immature enterocyte model), 2'-FL modulated similar signal molecules: CD14 and NF-κB induction was repressed, while IκB and SOCS2 expression and STAT3 phosphorylation were induced (figure 4C). Thus, 2'-FL modulated the same signalling pathways in models of immature and mature enterocytes.

2'-FL inhibits AIEC infection and inflammation in vivo

Three days of 0.25% DSS (a low dose) with streptomycin on the 4th day disrupts mouse microbiota. AIEC inoculation on the 5th day resulted in overt infection, manifesting as body weight loss (~10%) 3 days and 4 days post AIEC. 2'-FL by gavage once per day for the 4 days preceding inoculation prevented the body weight loss (figure 5A). AIEC infected mice had shortened

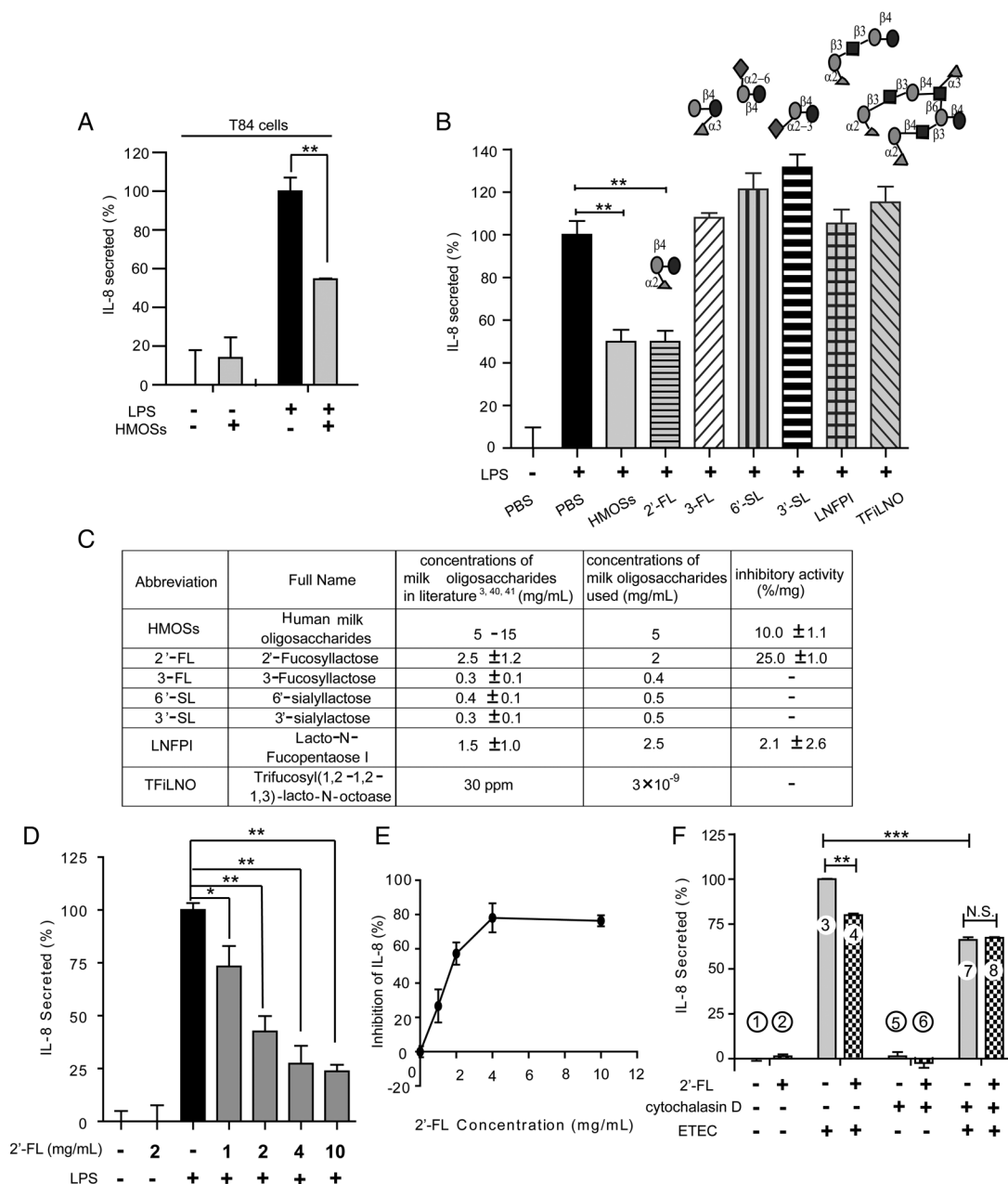


Figure 1 Lipopolysaccharide (LPS) induction of interleukin (IL)-8 is inhibited by 2'-fucosyllactose (2'-FL). T84 cells were treated with human milk oligosaccharide (HMOs) or its individual components for 48 hours and then challenged by LPS. The magnitude of the inflammatory response was measured as IL-8 levels; (A), (B) and (D) are normalised to untreated LPS response (100%) and (F) to untreated enterotoxigenic *E. coli* (ETEC) infection to facilitate comparison across independent experiments. (A) LPS-induced IL-8 secretion was inhibited by HMOs. (B) Isolated oligosaccharides of the HMOs mixture were tested individually at physiologically relevant concentrations for their ability to inhibit LPS induced IL-8 secretion. Schematic structures of the individual oligosaccharides are above each bar of the histogram: black circle, D-glucose; grey circle, D-galactose; black square, N-acetylglucosamine; triangle, L-fucose; dark grey diamond, N-acetyl-neuraminic acid. (C) Concentrations and inhibitory activities of the oligosaccharides 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 6'-SL, α 2,6 sialyllactose; 3'-SL, α 2,3 sialyllactose; lacto-N-fucopentaose I (LNFP I), Lacto-N-fucopentaose I; TFILNO, trifucosyl(α 1-2; α 1-2; α 1-3)-iso-lacto-N-octaose. (D and E) 2'-FL inhibition of IL-8 induction was dose dependent through 4 mg/mL. (F) 2'-FL specifically inhibits the IL-8 induction by ETEC invasion. Incubation of T84 cells with 2'-FL (4 mg/mL) for 48 hrs did not influence the basal expression of IL-8 (bars 1 and 2), but attenuated the ETEC induced IL-8 upregulation (bars 3 and 4). Incubation with cytochalasin D (2 μ M) did not affect the basal expression of IL-8 in the presence or absence of 2'-FL (bars 5 and 6). Cytochalasin D treatment of T84 cells for 30 minutes before ETEC infection (MOI=20) to inhibit invasion reduced induction of IL-8 after exposure to ETEC (bars 3 and 7). In these T84 cells whose invasion by ETEC was blocked by cytochalasin D, the residual IL-8 expression was not changed by treatment with 2'-FL (bars 7 and 8), indicating that 2'-FL inhibited only the IL-8 induced by ETEC invasion. Mean \pm SEM, n=6 for all experiments; * p ≤0.05; ** p ≤0.01; *** p ≤0.001 by analysis of variance. MOI, multiplicity of infection.

colons, but the lengths were closer to normal in AIEC inoculated mice pretreated with 2'-FL (figure 5B). Antibody against O83-antigen (expressed on LPS-positive AIEC LF82) revealed

less colonisation in the 2'-FL pretreated group (figure 5C). Cultures from fresh faeces (not shown) and colon confirmed that mice in the 2'-FL pretreated group were colonised less by

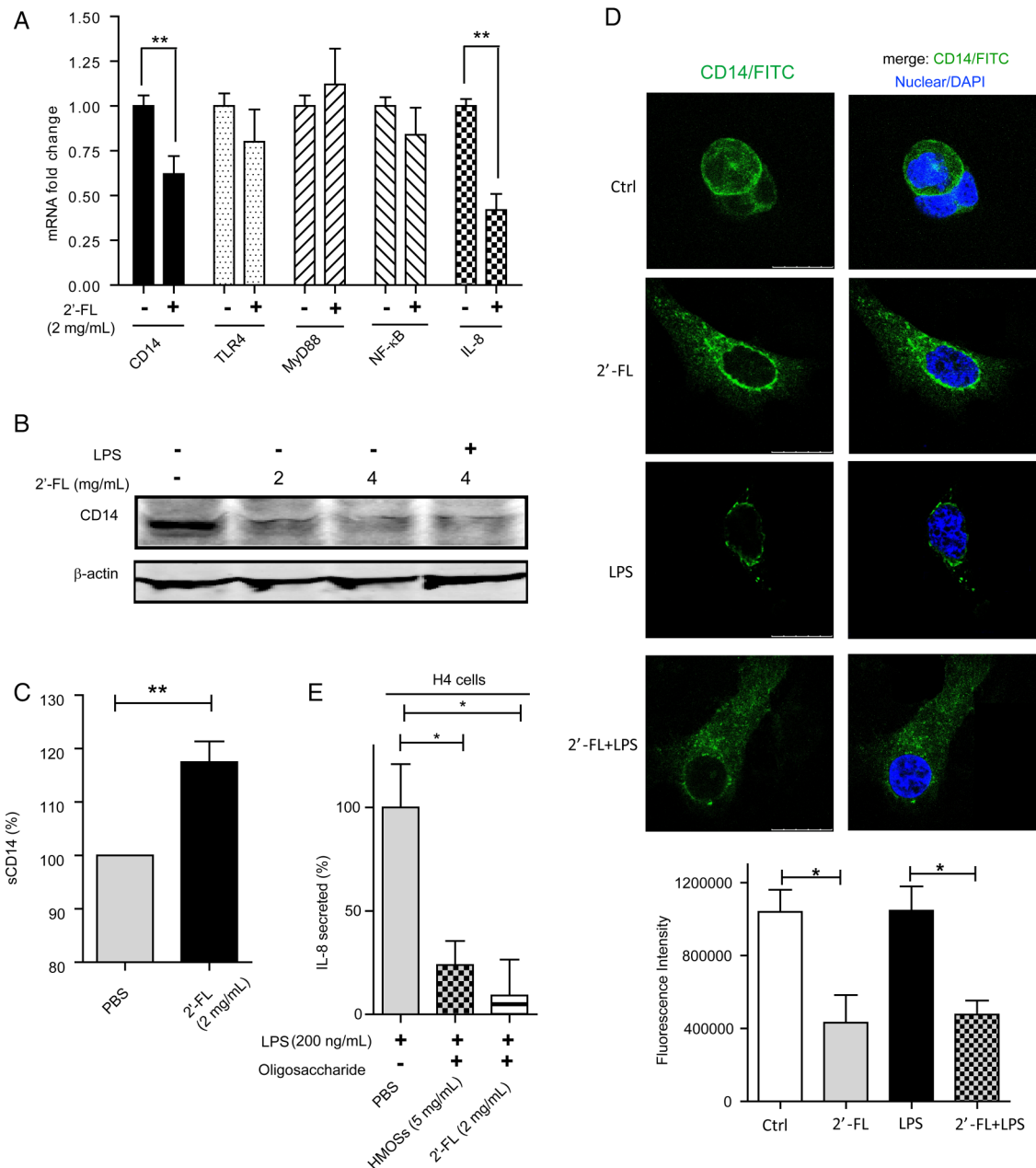


Figure 2 2'-fucosyllactose (2'-FL) alters CD14 expression and distribution in T84 cells. T84 cells were treated with 2'-FL for 48 h and then challenged by 100 μ g/mL lipopolysaccharide (LPS). Changes induced by 2'-FL of CD14 mRNA levels, protein levels and intracellular distribution were measured. H4 cells, a model of immature intestinal epithelial cells (IECs), are more sensitive to LPS; 200 ng/mL strongly induces interleukin (IL)-8 secretion. (A) In T84 cells treated with 2'-FL for 48 h, CD14 mRNA levels, measured by RT-PCR, decreased (n=6). (B) In T84 cells treated with 2'-FL, basal and LPS-stimulated CD14 protein expression, measured by western blot, decreased (representative of n=3). (C) After 48 h of incubation with 2'-FL, concentrations of soluble CD14 in T84 culture supernatants increased (n=6). (D) After incubation with 2'-FL, membrane-bound CD14 relocated into cytoplasm in basal and LPS-treated T84 cells (n=8). (E) H4 cells are more sensitive to LPS than T84 cells, and incubation with 2'-FL attenuates IL-8 induction more strongly, supporting clinical relevance of suppression of inflammation by human milk oligosaccharides (HMOs) and 2'-FL in the neonate. Means \pm SEM n=6; *p \leq 0.05; **p \leq 0.01 by analysis of variance.

inoculated AIEC (figure 5D). There was less AIEC-induced CD14 expression in colonic crypts of 2'-FL treated mice, (figure 5E), fewer CD14 positive cells in muscularis mucosa (not shown), and lower CD14-mRNA levels (figure 5F). H&E staining in colon tissue of AIEC-infected mice revealed epithelial cell sloughing, immune cell infiltration, and muscularis mucosa hyperplasia, while colons of 2'-FL pretreated mice exhibited fewer of these manifestations of inflammation (see online supplementary figure S7). AIEC infection was accompanied by elevated IL-6, IL-17 and TNF- α , major inflammatory cytokines of

mouse mucosa, and 2'-FL pretreatment inhibited this induction (figure 5G). IL-1 β , IFN- γ and IL-10 were not significantly affected by AIEC infection nor by 2'-FL (not shown).

DISCUSSION

Fucosylated moieties of the intestinal mucosa are targets for pathogens such as rotavirus A and *Campylobacter jejuni*.⁴⁵⁻⁴⁹ Fucosylation of the gut also provides anchors for mutualist microbes, and inability to produce these fucosylated anchors in α -1,2-fucosyltransferase 2 (FUT2) null non-secretors is

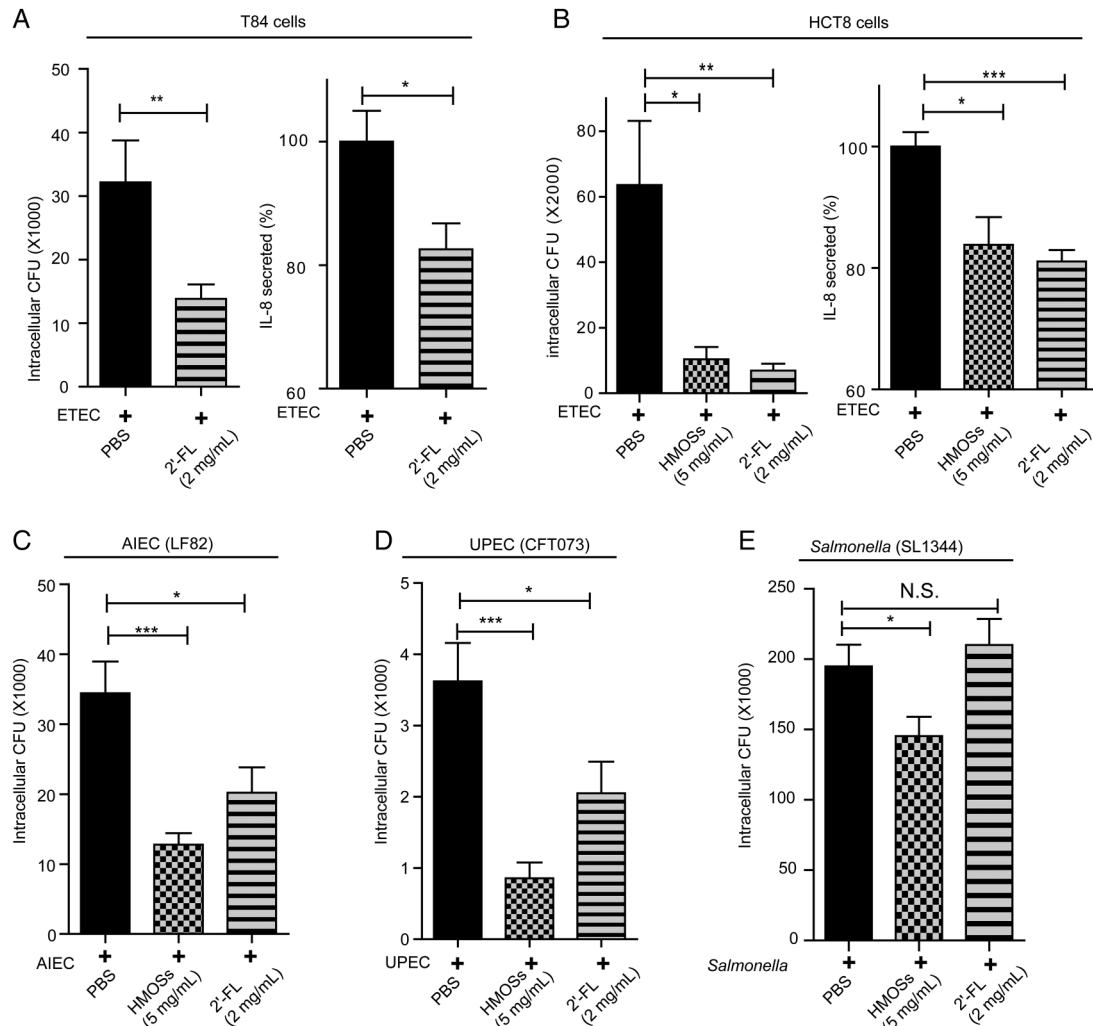


Figure 3 Interleukin (IL)-8 stimulation in other intestinal epithelial cells (IECs) and by different type 1 pili *Escherichia coli* is also attenuated by 2'-fucosyllactose (2'-FL). T84 or HCT8 cells were treated with 2'-FL or human milk oligosaccharide (HMOs) for 48 h and then challenged by enterotoxigenic *E. coli* (ETEC), adherent-invasive *E. coli* (AIEC), uropathogenic *E. coli* (UPEC) or *Salmonella enterica* serovar *Typhimurium*; bacterial invasion and IL-8 induction were measured. (A) 2'-FL inhibited ETEC invasion and IL-8 secretion in T84 IECs (derived from a colonic carcinoma). (B) HMOs and 2'-FL inhibited ETEC invasion and IL-8 secretion in HCT8 IECs (derived from ileal adenocarcinoma). (C) HMOs and 2'-FL inhibit AIEC (LF82, a type I pili *E. coli*) invasion in T84 cells; (D) HMOs and 2'-FL inhibit UPEC (CF073, a type I pili *E. coli*) invasion in T84 cells. (E) HMOs, but not 2'-FL, inhibit *Salmonella enterica* serovar *Typhimurium* (SL1344) invasion into T84 cells. *Salmonella* SL1344 invasion of T84 is not dependent on type I pili; therefore, the inability of 2'-FL to inhibit its invasion is consistent with 2'-FL specifically inhibiting type I pili mediated pathophysiology. The ability of HMOs to inhibit its invasion suggests that other components of the HMOs inhibit the type III secretion virulence factors of *Salmonella*. Mean±SEM, n=6 for all experiments; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ by analysis of variance.

associated with elevated risk of IBD, including Crohn's disease and UC, and type 1 diabetes. Indeed, the ontogeny of intestinal FUT2, which is regulated by microbiota, is requisite for gut homeostasis.⁵⁰ The presence of 2'-FL in human milk can modify the microbiota.⁵¹

In mammary gland, 2'-FL is synthesised through enzymatic transfer of fucose from guanosine 5'-diphosphate-l-fucose to lactose by FUT2.⁵² The resulting α -1,2-linked fucosyllactose, 2'-FL,⁵³ is the predominant oligosaccharide in the milk of secretors, representing up to 30% of total HMOs.^{54, 55} As with all HMOs, 2'-FL varies among individuals and over the course of lactation, and may occur in even higher concentrations in colostrum.⁵⁶

Two robust biological activities have been attributed to 2'-FL: First, by binding to adhesins of *Campylobacter jejuni*, *Salmonella typhi*, *Enterotoxigenic E. coli*, *Helicobacter pylori* and to capsids of noroviruses, 2'-FL inhibits binding of these

pathogens to their host receptors, the obligatory first step in their pathogenesis. Thus, 2'-FL decreases risk of diarrhoea in breastfed infants.^{3, 54, 55, 57, 58} Second, 2'-FL is prebiotic, a dietary carbohydrate resistant to digestion and absorption by the intestinal mucosa; in the distal gut, it stimulates growth of beneficial microbiota, including *Bifidobacterium bifidum*.⁵⁹ A third postulated activity is attenuation of inflammation. HMOs of colostrum quench major immunological pathways in immature human intestine ex vivo, and curb PAMP-associated stimulation. Particular pathways are attenuated by individual HMOs,¹⁸ however specific immunomodulation by 2'-FL had not been described.

The study herein describes a major inflammatory pathway attenuated specifically by 2'-FL. The LPS dependent induction of IL-8 expression caused by invasion of IECs by type 1 pili *E. coli* is inhibited by 2'-FL. 2'-FL attenuates cell associated expression of CD14, thereby quenching IL-8 release. The specificity of

Table 1 T84 cell proteins changed by 2'-FL

Gene ID	Ab-Ag	INR	Name
3611	ILK	0.0	integrin-linked kinase: regulates integrin-mediated signal transduction and mediates epithelial to mesenchymal transition
3903	LAIR-1	0.0	leukocyte-associated immunoglobulin-like receptor 1: regulates the immune response to prevent lysis of cell recognised as self
4478	Moesin	0.0	cell-cell recognition and motility signalling
3303	Hsp 70	0.6	heat shock 70 kDa protein 1A: stabilises proteins against aggregation and mediates folding of newly translated proteins
1398	Crk	0.7	v-crk sarcoma virus CT10 oncogene homologue (avian): binds tyrosine-phosphorylated proteins; attachment, membrane ruffling and motility
1195	Clk1 (Sty)	0.7	CDC-like kinase 1: phosphorylates serine/arginine-rich proteins during pre-mRNA nuclear processing
5159	PDGF receptor β	1.3	platelet-derived growth factor receptor, β polypeptide: mitogens for mesenchyme-derived cells
3486	IGFBP-3	1.3	insulin-like growth factor binding protein 3: prolongs the half life of IGFs and alters their interaction with cell surface receptors
5575	PKA RI	1.3	cAMP-dependent protein kinase (regulatory, type I): controls regulation of metabolism, ion transport and gene transcription
2237	FEN-1	1.3	flap structure-specific endonuclease 1:1 of 10 proteins essential for cell-free DNA replication
4288	Ki-67	1.4	antigen identified by monoclonal antibody Ki-67: associated with cellular proliferation
1294	COL7A1	1.4	Collagen (type VII, α 1): anchoring fibril between the external epithelium and the underlying stroma
995	CDC25C	1.5	Cell division cycle 25C: regulates cell division by directing dephosphorylation of cyclin B bound CDC2 triggering entry into mitosis
3837	Karyopherin β	1.5	karyopherin (importin) β 1: component of nucleocytoplasmic transport complex
6595	Brm	1.5	SMARCA2, similar to the brahma protein of Drosophila: helicase and ATPase activity; transcriptional activation
5705	PSMC5	1.5	Proteasome, macropain 26S subunit (ATPase, 5): cleaves MHC1 peptides in ATP/ubiquitin-dependent (non-lysosomal) process
5588	PKC θ	1.6	protein kinase C (θ): calcium independent protein kinase activation of T cells, NF- κ B and AP-1
10507	CD100	1.6	Cluster of differentiation 100: binds CD72; activate B cells and dendritic cells
4842	nNOS	1.6	nitric oxide synthase 1: nitric oxide release from L-arginine; mediates neurotransmission; antimicrobial and antitumor activity
7157	p53	1.6	tumor protein p53: cell stress mediator activates genes of cell cycle arrest, apoptosis, senescence, DNA repair and metabolic changes
9044	BTAf1	1.7	B-TATA box-binding protein-associated factor: associates with TBP (TATA box-binding protein) to form B-TFIID complex to initiate transcription of genes by RNA polymerase II
51272	BET1L	1.7	Bet1 Golgi vesicular membrane trafficking protein-like: targeting and fusion of retrograde transport vesicles with the Golgi complex; integrity of the Golgi complex
84254	CAMKK1	1.8	calcium/calmodulin-dependent protein kinase kinase 1, alpha: calcium/calmodulin-dependent (CaM) kinase cascade
6908	TBP	1.8	TATA box binding protein: initiation of transcription by RNA polymerase II
4830	NME1	1.9	NME/NM23 nucleoside diphosphate kinase 1: reduces mRNA transcript levels in highly metastatic cells
10399	RACK1 (GNB2L1)	2.3	Receptor for activated protein kinase C-1 (guanine nucleotide binding protein 2L1): multifaceted scaffolding protein; development, cell migration, central nervous system function, circadian rhythm.
3710	IP3R-3	2.5	inositol 1,4,5-triphosphate receptor, type 3: exocrine secretion, energy metabolism and growth
4687	NCF1C	3.0	neutrophil cytosolic factor 1 (pseudogene): 47 kDa cytosolic subunit of neutrophil NADPH oxidase that produces superoxide anion.

2'-FL, 2'-fucosyllactose; CDC, cell division cycle; INR, internally normalised ratio.

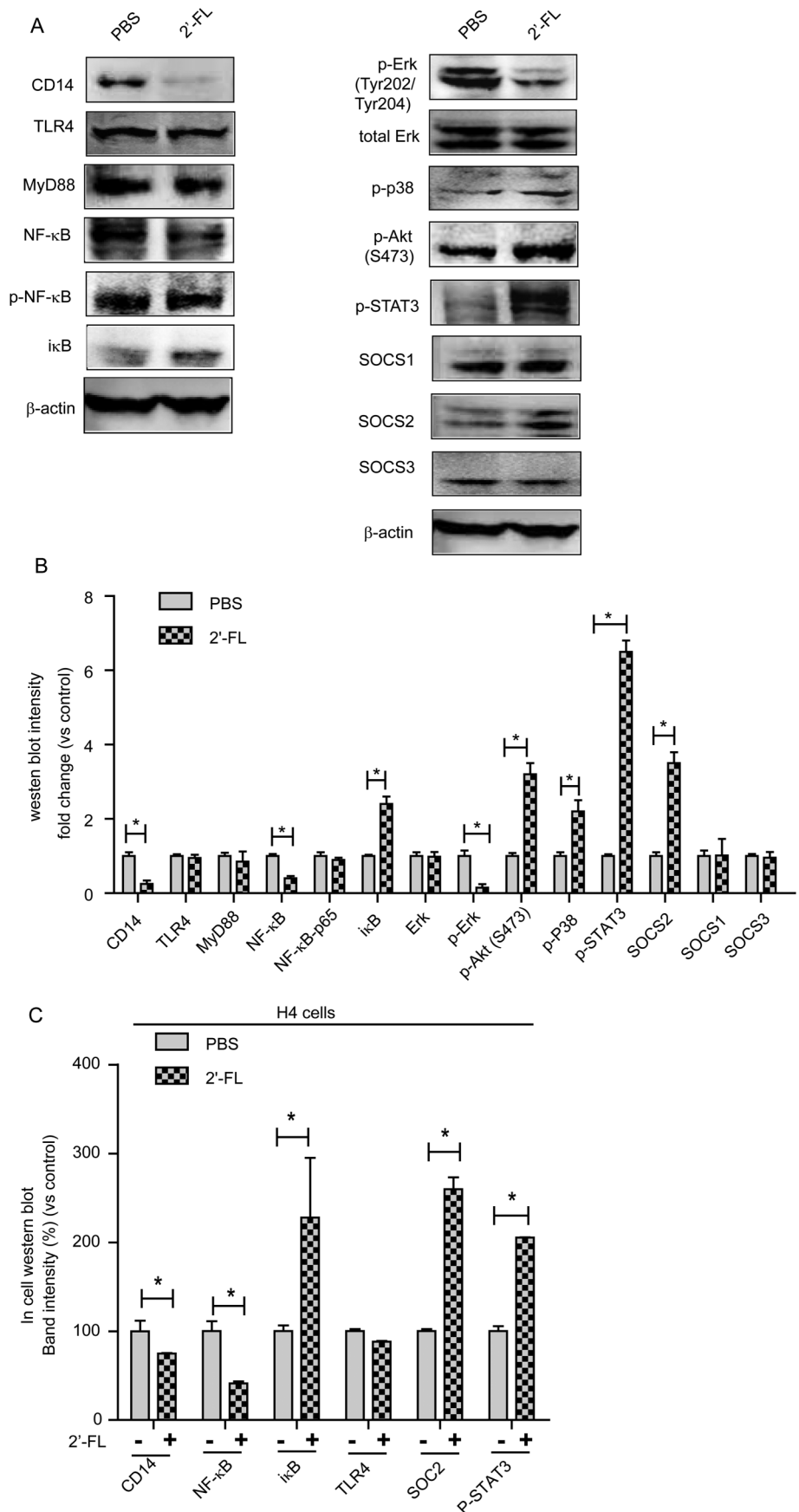
this inhibition is illustrated by the inability of physiological concentrations of other HMOs, including 6'-SL, 3'-SL, LNFP I and TFILNO, or even 3-FL, a positional isomer of 2'-FL, to exhibit similar anti-inflammatory activity at their physiological concentrations (figure 1C). These results are consistent with 2'-FL modulation of CD14 expression requiring precise structural recognition by IECs. Moreover, 2'-FL inhibits only the increased IL-8 induced by infection, as evident by the inability of 2'-FL to inhibit basal IL-8 production, or IL-8 production in ETEC exposed cells in which invasion per se is inhibited. The ability of 2'-FL to inhibit inflammation associated with *E. coli* invasion was confirmed in AIEC-infected mice. Combined with the previous observation that galactosyl lactoses specifically inhibit polyinosinic-polycytidylic acid-induced signalling pathways,¹⁸ the data indicate that individual oligosaccharides may modulate distinct inflammatory pathways. The signalling cascade specifically modulated by 2'-FL is illustrated in figure 6. Treatment of IECs with 2'-FL increased expression of SOCS2 and phosphorylation of STAT3, and attenuated transcription and translation of cell-associated CD14, thereby quenching the typical IL-8 response of IECs to infection. 2'-FL likewise modulates IL-8 induction by exogenous LPS via identical changes in signalling.

CD14 plays a central role in TLR4 recognition of LPS; these three molecules form a tripartite complex that activates

signalling pathways that induce production of inflammatory mediators such as IL-8.^{60 61} CD14 exists in a membrane form (mCD14) and soluble forms (sCD14),⁶² with each form having multiple activities. mCD14 is a GPI-anchored membrane protein⁶² whose expression level and location change during intestinal development and inflammatory disease.^{61 63} mCD14 is the co-receptor of LPS, and mediates cellular trafficking, such as phagocytosis of invasive bacteria and elimination of mammalian bodies during cellular apoptosis.^{64 65} Due to mCD14 being a co-receptor of LPS binding to TLR4, the reduction of mCD14 expression, such as by IL-4 and IL-13, is anti-inflammatory.⁶¹ Formation of the mCD14-TLR4 complex induces trafficking: mCD14 promotes LPS-induced endocytosis of TLR4, and mCD14 and TLR4 traffic together into the endosome.⁶⁶ Internalisation of TLR4 may truncate inflammatory reactions⁶⁷; this would serve to keep TLR4-mediated inflammatory responses pulsatile rather than chronic. Low levels of mCD14 reflect its loss during internalisation of TLR4, an anti-inflammatory state; conversely, the low TLR4 internalisation during inflammation would increase levels of mCD14. Note that abnormally high expression of mCD14 is associated with IBD.^{61 68}

sCD14 is naturally present in normal human serum, milk and cell culture supernatants.^{60 61 69} sCD14 can have two forms.⁷⁰

Figure 4 2'-fucosyllactose (2'-FL) treatment modifies signalling pathway intermediates. T84 cells were treated with 2'-FL for 48 h, proteins extracted, and signalling molecules measured by western blot. (A) 2'-FL modulates signalling intermediates of cytokine production in T84 cells. (B) Quantitative comparison of western blot band intensity from T84 cells (n=3). (C) H4 cells were plated at 5000 cells/well, treated with 2'-FL, and signalling proteins were measured by in-cell (in situ) western blot. H4 cell expression of signalling mediators were modulated by 2'-FL, confirming that the signalling changes observed in T84 cells are relevant to immature enterocytes (n=6). Mean \pm SEM; * $p \leq 0.05$ by analysis of variance.



The 48 kDa sCD14 is a product of serine protease cleavage of mCD14.⁶¹ The 56 kDa sCD14 results from retention of the C-terminal sequence previously attached to GPI.^{61 71} They are

considered collectively as sCD14, as the relative activities of these two forms are not clear. mCD14 is a more efficient activator of TLR4 signalling than sCD14. Endothelial cells express

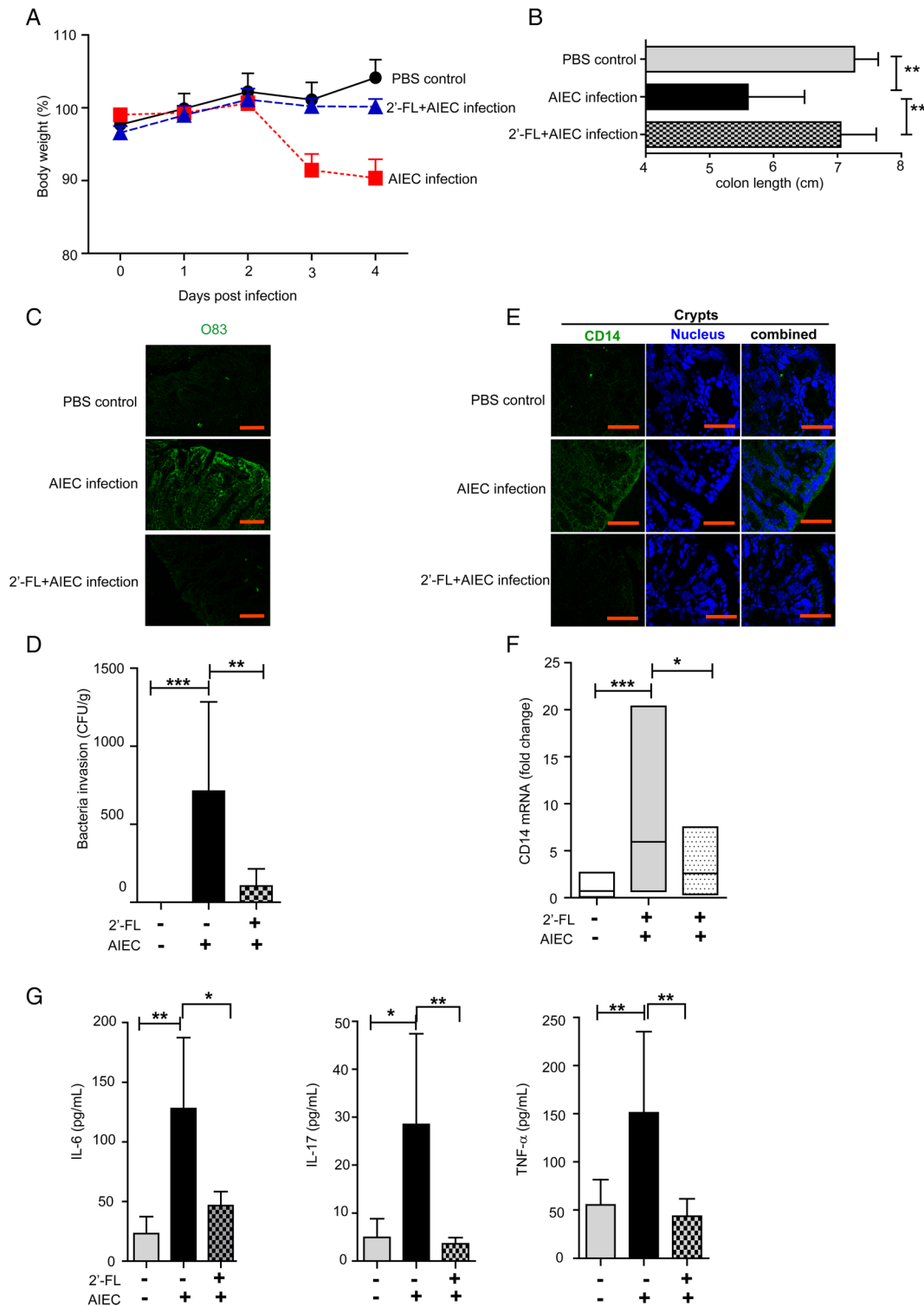


Figure 5 2'-fucosyllactose (2'-FL) modulates CD14 expression and attenuates inflammation in adherent-invasive *E. coli* (AIEC) infected mice. C57BL/6 mice received 0.25% dextran sodium sulfate (DSS) in their drinking water for 3 days, and were given 20 mg of streptomycin by gavage on day 4; half also received 100 mg of 2'-FL in 200 μ L by gavage for each of the 4 days. On the 5th day, the two groups of experimental mice were inoculated with 10^9 colony forming unit (CFU) AIEC via gavage and sacrificed after 4 days; a control group received DSS and antibiotic, but only a sham PBS inoculum. (A) The body weight loss that follows AIEC infection did not occur with 2'-FL pretreatment. (B) The reduction in colon length caused by AIEC infection did not occur with 2'-FL pretreatment. (C) 2'-FL pretreatment inhibited the colonisation of the colonic mucosa by O83-positive bacteria. Representative images from six mice; orange bar=50 μ m. (D) Antibiotic washed colon cultured fewer AIECs from 2'-FL pretreated mice. (E) CD14 expression was lower in colonic crypts cells of 2'-FL pretreated mice. (Confocal microscopy, orange bars = 50 μ m). Images are representative of six mice. (F) CD14 mRNA levels in colon were reduced by 2'-FL pretreatment. (G) Interleukin (IL)-6, IL-17 and TNF- α levels were lower in colon of 2'-FL pretreated mice. Mean \pm SEM, n=6 for all groups; *p \leq 0.05; **p \leq 0.01; ***p \leq 0.001 by analysis of variance.

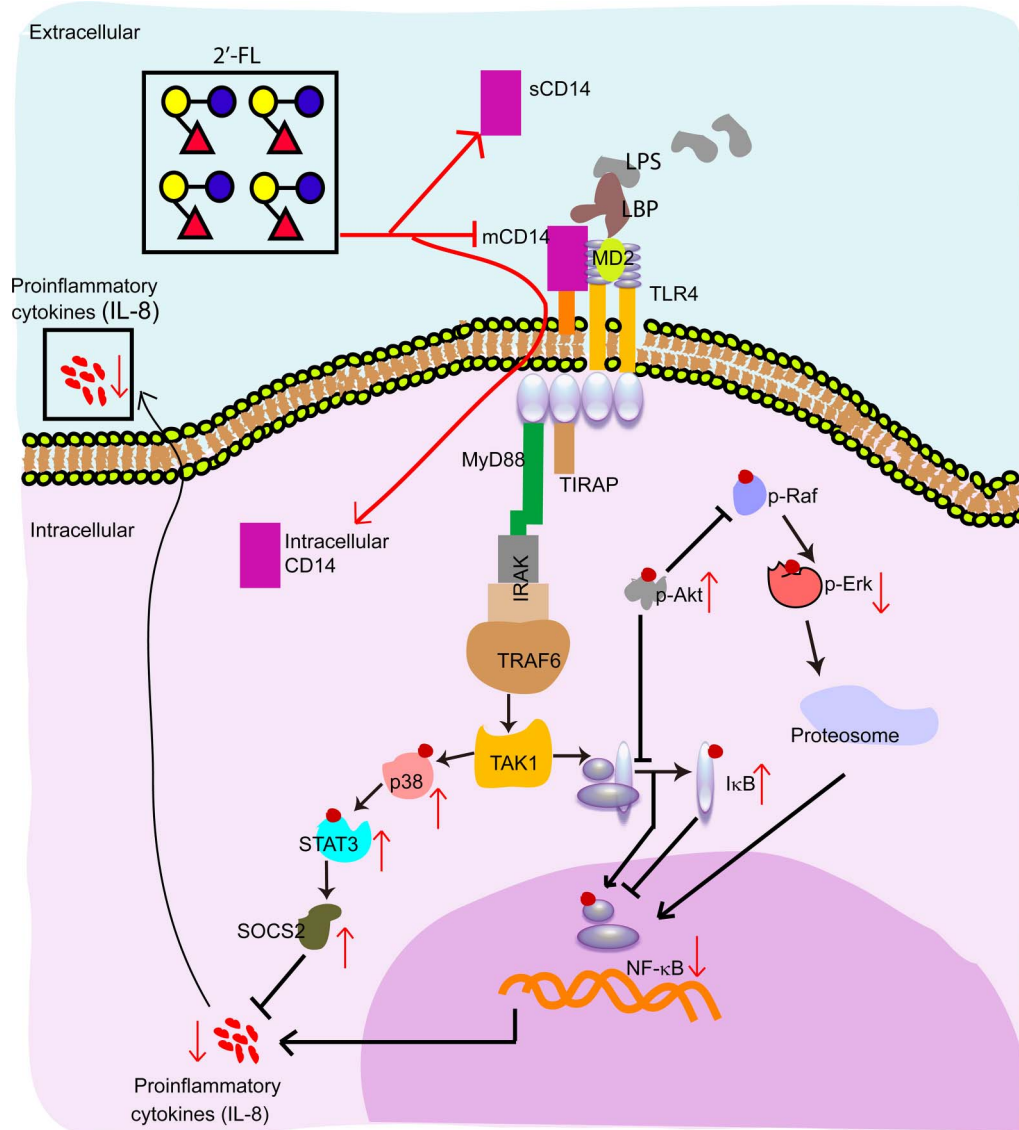


Figure 6 Proposed signalling pathway modulation by 2'-fucosyllactose (2'-FL). 2'-FL inhibits membrane-bound CD14 expression, while increasing (soluble form of CD14) sCD14 in the supernatant and the internalisation of membrane form of CD14 (mCD14). 2'-FL exposure increases the relative amounts of negative regulatory molecules, including p-Akt, p-p38, suppressors of cytokine signalling 2 (SOCS2), phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and IκB, while repressing pErk and NF-κB levels. Red lines connect signalling molecules whose levels change upon exposure to 2'-FL.

TLR4 but are intrinsically CD14-negative; therefore circulating sCD14 initiates TLR4 inflammatory signalling.⁶¹ Immature IECs also express little CD14 relative to that in mature cells. Therefore, sCD14 in human milk, when bound to the LPS of Gram-negative bacteria in the infant intestinal tract, is proposed to be a 'sentinel' molecule that activates TLR4-mediated inflammatory signalling.⁷² In contrast to these local proinflammatory effects, circulating sCD14 exhibits anti-inflammatory effects in tissues distant from the inflammation.⁷³ In these mCD14 positive tissues, circulating sCD14 competes with mCD14, promotes internalisation of TLR4, thereby decreasing the TLR4 response to LPS⁷⁴ and limiting the intensity and duration of the inflammatory response. The effects of 2'-FL on CD14 occur at multiple levels: 2'-FL decreases levels of cell-associated CD14 by suppression of CD14 transcription and translation (figure 2A, B); 2'-FL induces endocytosis of surface mCD14 into the cytoplasm (figure 2D); and 2'-FL increases the secretion of sCD14 to extracellular culture supernatants (figure 2C). Each

of these could contribute to the quenching of inflammatory activity by 2'-FL.

Downstream TLR4-LPS-CD14 activation of several major signalling molecules was modulated by 2'-FL, summarised in figure 6. Most mediate either the Akt or the SOCS2 pathway within the MIF network. The phosphatidylinositol 3-kinase/Akt pathway limits LPS activation of signalling pathways.^{75 76} pAkt blocks pNF-κB and pRaf.⁷⁵ pRaf promotes p-Erk and induces NF-κB.⁷⁵ Thus, the striking upregulation of pAkt by 2'-FL represses pErk and NF-κB, an anti-inflammatory effect that is consistent with the experimental data.

SOCS proteins are negative regulators of cytokine signalling. They are activated most commonly through the janus kinase and STAT pathways.^{77 78} SOCS2 (figure 4A,C), as well as its upstream signalling molecules p-p38 and pSTAT3 (figures 4A,B and 6), are upregulated by 2'-FL. SOCS2-inhibited signalling molecules are thought to regulate growth more than immune function.⁷⁹ However, the data herein, in which 2'-FL

upregulates SOCS2 while quenching IL-8 production, suggest that SOCS2 may suppress mediators of LPS-induced IL-8 activation. 2'-FL stimulation of SOCS2 could also promote maturation of immature epithelial cells through signalling molecules related to proliferation (Ki-67) and cell cycle (CDC25C), an independent more indirect mechanism to attenuate inflammation. This phenomenon may be addressed in further studies.

2'-FL suppression of cell-associated CD14 expression prevents LPS-dependent type 1 pili *E. coli* invasion while modulating the inflammation induced by LPS at the mucosal surface. This ability of 2'-FL to quench LPS stimulated inflammation may be of clinical relevance for neonates. Relative to mature intestinal mucosa, more TLR4 is on the cell surface of the immature mucosa, and this may be linked to the hypersensitivity to inflammatory stimuli exhibited by developing intestine.⁴² This hypersensitivity creates a challenge during initial colonisation, where the innate immune system is naïve to all microbes. Although Gram-negative (LPS-positive) *E. coli* may represent an especially potent inflammatory stimulus,⁸⁰ exposure to exogenous endotoxin causes IECs to acquire TLR tolerance immediately after birth. This facilitates microbial colonisation by mutualist and commensal bacteria, allowing development of a stable intestinal host-microbe mutualism⁸¹; imbalance leads to dysbiosis, increasing risk of NEC.⁸² If the 2'-FL prevalent in most human milk is a major factor in quenching inflammation during this transition from fetal to colonised postnatal intestinal mucosa, 2'-FL should inhibit inflammation in most IEC models.

2'-FL suppressed inflammatory signalling in two additional cell culture IEC models. HCT8 is more sensitive to inflammatory stimuli, and closer than T84 to the physiology of IECs in situ. The H4 line, derived from immature intestine, recapitulates many IEC characteristics of immature intestine. Pretreatment of either cell line with 2'-FL induced resistance to ETEC invasion and inhibition of IL-8 induction similar to that observed in T84 cells (see online supplementary figure S1, S2 and S4). The ability of 2'-FL to inhibit AIEC colonisation was confirmed in vivo, measured as lower AIEC in faeces and colon following a standard inoculum. In AIEC-infected mice, 2'-FL pretreatment prevented body weight loss, and quenched inflammation through repression of CD14 expression. AIEC induction of proinflammatory cytokine levels was prevented by 2'-FL, resulting in lower histopathology inflammatory scores and other signs of colon inflammation (figure 5).

Moreover, 2'-FL inhibits multiple type 1 pili invasive *E. coli*: the primary model ETEC strain, UPEC and AIEC. Type 1 pili *E. coli* are major pathogens causing millions of cases annually of urinary (UPEC) or gastroenterological (ETEC) infection,^{83–84} and AIEC are associated with IBD.^{85–88} Inhibition of *E. coli* pathogenesis by 2'-FL may underlie the association between breast feeding and decreased risk of disease, and suggests a novel treatment for type 1 pili *E. coli*-associated enteric and urinary tract infections.⁸⁹ Moreover, the inhibition of salmonella infection by HMOs but not by 2'-FL implies that HMOs contain other oligosaccharides that protect against pathogens whose critical determinants are distinct from type 1 pili.

Breast feeding is associated with decreased risk of inflammatory infectious diseases, and 2'-FL is a principal human milk component. 2'-FL alone inhibits proinflammatory signalling induced by type 1 pili *E. coli* pathogen invasion (or by LPS) by suppressing CD14 expression in IECs. This quenching of inflammation by 2'-FL is additional evidence that HMOs comprise a milk-borne innate immune system through which the mother protects her infant from environmental insult while the infant's immature mucosal immune system is developing and

maturing. But this is only one oligosaccharide and one pathway; 2'-FL and other oligosaccharides of human milk could influence additional mucosal signalling pathways, thereby contributing towards the orderly maturation and colonisation of the infant gut. HMOs may represent a fresh category of prophylactic and therapeutic agents, and 2'-FL might be an early candidate for clinical trials against LPS-mediated inflammation.

Acknowledgements The authors thank Paul McCabe for assistance with editing the manuscript.

Contributors YYH, SBL and DSN designed the research; YYH, SBL, DEK, SL, NTL, YH, SBF and DRH conducted the experiments; YYH, SBL and DSN analysed the data; YYH and DSN wrote the first draft of the manuscript and all authors contributed to its completion.

Funding Partially supported by NIH grants R01HD059140, U01AI075563 and P01HD013021 and Abbott Nutrition.

Competing interests DSN owns stock in Glycosyn, which makes human milk oligosaccharides. This is being managed by Boston College. Other authors have no competing interests to declare.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

- Klement E, Cohen RV, Boxman J, *et al.* Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis. *Am J Clin Nutr* 2004;80:1342–52.
- Buescher ES. Anti-inflammatory characteristics of human milk. In: Newburg D, ed. *Bioactive components of human milk*. Springer US, 2001:207–22.
- Newburg DS, Ruiz-Palacios GM, Morrow AL. Human milk glycans protect infants against enteric pathogens. *Annu Rev Nutr* 2005;37:5–58.
- Kunz C, Rudloff S, Baier W, *et al.* Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr* 2000;20:699–722.
- Sela DA, Mills DA. Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides. *Trends Microbiol* 2010;18:298–307.
- Urashima T, Saito T, Nakamura T, *et al.* Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconj J* 2001;18:357–1.
- Newburg DS. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. *J Anim Sci* 2009;87:26–34.
- Kunz C, Rudloff S, Baier W, *et al.* Oligosaccharides in human milk: Structural, functional, and metabolic aspects. *Annu Rev Nutr* 2000;20:699–722.
- Chichlowski M, German JB, Lebrilla CB, *et al.* The influence of milk oligosaccharides on microbiota of infants: opportunities for formulas. *Annu Rev Food Sci Technol* 2011;2:331–51.
- Bode L. Human milk oligosaccharides: prebiotics and beyond. *Nutr Rev* 2009;67: S183–S91.
- Schumacher G, Bendas G, Stahl B, *et al.* Human milk oligosaccharides affect P-selectin binding capacities: In vitro investigation. *Nutrition* 2006;22:620–7.
- Bode L, Kunz C, Muhly-Reinholz M, *et al.* Inhibition of monocyte, lymphocyte, and neutrophil adhesion to endothelial cells by human milk oligosaccharides. *Thromb Haemost* 2004;92:1402–10.
- Vos AP, M'Rabet L, Stahl B, *et al.* Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. *Crit Rev Immunol* 2007;27:97–140.
- Velupillai P, Harn DA. Oligosaccharide-specific induction of interleukin 10 production by B220+ cells from schistosoma-infected mice: a mechanism for regulation of CD4+ T-cell subsets. *Proc Natl Acad Sci USA* 1994;91:18–22.
- Terrazas LI, Walsh KL, Piskorska D Jr., *et al.* The schistosome oligosaccharide lacto-N-neotetraose expands Gr1(+) cells that secrete anti-inflammatory cytokines and inhibit proliferation of naive CD4(+) cells: a potential mechanism for immune polarization in helminth infections. *J Immunol* 2001;167:5294–303.
- Eiwegger T, Stahl B, Haidl P, *et al.* Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. *Pediatr Allergy Immunol* 2010;21:1179–88.
- Lane JA, O'Callaghan J, Carrington SD, *et al.* Transcriptional response of HT-29 intestinal epithelial cells to human and bovine milk oligosaccharides. *Br J Nutr* 2013;111–11.
- He Y, Liu S, Leone S, *et al.* Human colostrum oligosaccharides modulate major immunologic pathways of immature human intestine *Mucosal Immunol* 2014;7:1326–39.
- Makhseed M, Raghupathy R, Azizieh F, *et al.* Th1 and Th2 cytokine profiles in recurrent aborters with successful pregnancy and with subsequent abortions. *Hum Reprod* 2001;16:2219–26.
- Janeway CA Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197–216.

- 21 Ausubel FM. Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol* 2005;6:973–9.
- 22 Mannick E, Udall JN Jr., Neonatal gastrointestinal mucosal immunity. *Clin Perinatol* 1996;23:287–304.
- 23 Bailey M, Haverson K, Inman C, *et al.* The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effector function. *Proc Nutr Soc* 2005;64:451–7.
- 24 Beatty WL, Meresse S, Gounon P, *et al.* Trafficking of Shigella lipopolysaccharide in polarized intestinal epithelial cells. *J Cell Biol* 1999;145:689–8.
- 25 Trent MS, Stead CM, Tran AX, *et al.* Diversity of endotoxin and its impact on pathogenesis. *J Endotoxin Res* 2006;12:205–23.
- 26 Erridge C, Bennett-Guerrero E, Poxton IR. Structure and function of lipopolysaccharides. *Microbes Infect* 2002;4:837–51.
- 27 Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine* 2008;42:145–1.
- 28 Shimazu R, Akashi S, Ogata H, *et al.* MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999;189:1777–82.
- 29 Ulevitch RJ, Tobias PS. Recognition of gram-negative bacteria and endotoxin by the innate immune system. *Curr Opin Immunol* 1999;11:19–22.
- 30 Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. *Curr Opin Immunol* 2002;14:103–10.
- 31 Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat Rev Immunol* 2013;13:790–801.
- 32 Schilling JD, Mulvey MA, Vincent CD, *et al.* Bacterial invasion augments epithelial cytokine responses to Escherichia coli through a lipopolysaccharide-dependent mechanism. *J Immunol* 2001;166:1148–55.
- 33 Szebeni B, Veres G, Dezsöfi A, *et al.* Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol* 2008;151:34–41.
- 34 Kawada M, Chen CC, Arihiro A, *et al.* Chitinase 3-like-1 enhances bacterial adhesion to colonic epithelial cells through the interaction with bacterial chitin-binding protein. *Lab Invest* 2008;88:883–95.
- 35 Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* 2003;31:e154.
- 36 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 2001;25:402–8.
- 37 Martínez-Medina M, Denizot J, Dreux N, *et al.* Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut* 2014;63:116–24.
- 38 Small CL, Reid-Yu SA, McPhee JB, *et al.* Persistent infection with Crohn's disease-associated adherent-invasive Escherichia coli leads to chronic inflammation and intestinal fibrosis. *Nat Commun* 2013;4:1957.
- 39 Crane JK, Azar SS, Stam A, *et al.* Oligosaccharides from human milk block binding and activity of the Escherichia coli heat-stable enterotoxin (STa) in T84 intestinal cells. *J Nutr* 1994;124:2358–64.
- 40 Asakuma S, Akahori M, Kimura K, *et al.* Sialyl oligosaccharides of human colostrum: changes in concentration during the first three days of lactation. *Biosci Biotechnol Biochem* 2007;71:1447–51.
- 41 Asakuma S, Urashima T, Akahori M, *et al.* Variation of major neutral oligosaccharides levels in human colostrum. *Eur J Clin Nutr* 2008;62:488–94.
- 42 Nanthakumar N, Meng D, Goldstein AM, *et al.* The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS ONE* 2011;6:e17776.
- 43 Velge P, Wiedemann A, Rosselin M, *et al.* Multiplicity of Salmonella entry mechanisms, a new paradigm for Salmonella pathogenesis. *Microbiologyopen* 2012;1:243–58.
- 44 Anderson K, Potter A, Baban D, *et al.* Protein expression changes in spinal muscular atrophy revealed with a novel antibody array technology. *Brain* 2003;126:2052–64.
- 45 Ruiz-Palacios GM, Cervantes LE, Ramos P, *et al.* Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem* 2003;278:14112–20.
- 46 Smyth DJ, Cooper JD, Howson JM, *et al.* FUT2 nonsecretor status links type 1 diabetes susceptibility and resistance to infection. *Diabetes* 2011;60:3081–4.
- 47 Parmar AS, Alakulppi N, Paavola-Sakki P, *et al.* Association study of FUT2 (rs601338) with celiac disease and inflammatory bowel disease in the Finnish population. *Tissue Antigens* 2012;80:488–93.
- 48 Imbert-Marcille BM, Barbe L, Dupe M, *et al.* A FUT2 gene common polymorphism determines resistance to rotavirus A of the P[8] genotype. *J Infect Dis* 2014;209:1227–30.
- 49 Maroni L, van de Graaf SF, Hohenester SD, *et al.* Fucosyltransferase 2: a genetic risk factor for primary sclerosing cholangitis and Crohn's disease—a comprehensive review. *Clin Rev Allergy Immunol* 2014.
- 50 Nanthakumar NN, Dai D, Meng D, *et al.* Regulation of intestinal ontogeny: effect of glucocorticoids and luminal microbes on galactosyltransferase and trehalase induction in mice. *Glycobiology* 2005;15:221–32.
- 51 Yu ZT, Chen C, Kling DE, *et al.* The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology* 2013;23:169–77.
- 52 Isozaki T, Ruth JH, Amin MA, *et al.* Fucosyltransferase 1 mediates angiogenesis, cell adhesion and rheumatoid arthritis synovial tissue fibroblast proliferation. *Arthritis Res Ther* 2014;16:R28.
- 53 Kuhn R, Baer HH, Gauhe A. Fucosido-lactose, das Trisaccharid der Frauenmilch. *Chemische Berichte* 1955;88:1135–46.
- 54 Chaturvedi P, Warren CD, Altaye M, *et al.* Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* 2001;11:365–72.
- 55 Morrow AL, Ruiz-Palacios GM, Altaye M, *et al.* Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. *J Pediatr* 2004;145:297–303.
- 56 Coppa GV, Pierani P, Zampini L, *et al.* Oligosaccharides in human milk during different phases of lactation. *Acta Paediatr Suppl* 1999;88:89–94.
- 57 Chessa D, Winter MG, Jakomin M, *et al.* Salmonella enterica serotype Typhimurium Std fimbriae bind terminal alpha(1,2)fucose residues in the cecal mucosa. *Mol Microbiol* 2009;71:864–75.
- 58 Magalhaes A, Reis CA. Helicobacter pylori adhesion to gastric epithelial cells is mediated by glycan receptors. *Braz J Med Biol Res* 2010;43:611–8.
- 59 Lee WH, Pathanibul P, Quarterman J, *et al.* Whole cell biosynthesis of a functional oligosaccharide, 2'-fucosyllactose, using engineered Escherichia coli. *Microb Cell Fact* 2012;11:48.
- 60 Labeta MO, Vidal K, Norez JE, *et al.* Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. *J Exp Med* 2000;191:1807–12.
- 61 Antal-Szalmas P. Evaluation of CD14 in host defence. *Eur J Clin Invest* 2000;30:167–9.
- 62 Pugin J, Kravchenko VV, Lee JD, *et al.* Cell activation mediated by glycosylphosphatidylinositol-anchored or transmembrane forms of CD14. *Infect Immun* 1998;66:1174–80.
- 63 Goyert SM, Ferrero EM, Seremetis SV, *et al.* Biochemistry and expression of myelomonocytic antigens. *J Immunol* 1986;137:3909–14.
- 64 Devitt A, Moffatt OD, Raykundalia C, *et al.* Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* 1998;392:505–9.
- 65 Grunwald U, Fan X, Jack RS, *et al.* Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism. *J Immunol* 1996;157:4119–25.
- 66 Zanon I, Ostuni R, Marek LR, *et al.* CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 2011;147:868–0.
- 67 Yoshioka N, Taniguchi Y, Yoshida A, *et al.* Intracellular localization of CD14 protein in intestinal macrophages. *Anticancer Res* 2009;29:865–9.
- 68 Frolova L, Drastich P, Rossmann P, *et al.* Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. *J Histochem Cytochem* 2008;56:267–4.
- 69 Field CJ. The immunological components of human milk and their effect on immune development in infants. *J Nutr* 2005;135:1–4.
- 70 Labeta MO, Durieux JJ, Fernandez N, *et al.* Release from a human monocyte-like cell line of two different soluble forms of the lipopolysaccharide receptor, CD14. *Eur J Immunol* 1993;23:2144–51.
- 71 Buefler P, Stiegler G, Schuchmann M, *et al.* Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants. *Eur J Immunol* 1995;25:604–10.
- 72 Vidal K, Labeta MO, Schiffrin EJ, *et al.* Soluble CD14 in human breast milk and its role in innate immune responses. *Acta Odontol Scand* 2001;59:330–4.
- 73 Kitchens RL, Thompson PA. Modulatory effects of sCD14 and LBP on LPS-host cell interactions. *J Endotoxin Res* 2005;11:225–9.
- 74 Troelstra A, Giepmans BN, Van Kessel KP, *et al.* Dual effects of soluble CD14 on LPS priming of neutrophils. *J Leukoc Biol* 1997;61:173–8.
- 75 Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocyte cells. *J Biol Chem* 2002;277:32124–32.
- 76 Schabbauer G, Tencati M, Pedersen B, *et al.* PI3K-Akt pathway suppresses coagulation and inflammation in endotoxemic mice. *Arterioscler Thromb Vasc Biol* 2004;24:1963–9.
- 77 Alexander WS. Suppressors of cytokine signalling (SOCS) in the immune system. *Nat Rev Immunol* 2002;2:410–6.
- 78 Krebs DL, Hilton DJ. SOCS proteins: negative regulators of cytokine signaling. *Stem Cells* 2001;19:378–7.
- 79 Miller ME, Michaylira CZ, Simmons JG, *et al.* Suppressor of cytokine signaling-2: a growth hormone-inducible inhibitor of intestinal epithelial cell proliferation. *Gastroenterology* 2004;127:570–81.
- 80 Guarner F, Malagelada JR. Gut flora in health and disease. *Lancet* 2003;361:512–9.
- 81 Renz H, Brandtzaeg P, Hornef M. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. *Nat Rev Immunol* 2012;12:9–23.

- 82 Morrow AL, Lagomarcino AJ, Schibler KR, *et al.* Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome* 2013;1:13.
- 83 Fleckenstein JM, Hardwidge PR, Munson GP, *et al.* Molecular mechanisms of enterotoxigenic *Escherichia coli* infection. *Microbes Infect* 2010;12:89–98.
- 84 Shaikh N, Morone NE, Bost JE, *et al.* Prevalence of urinary tract infection in childhood: a meta-analysis. *Pediatr Infect Dis J* 2008;27:302–8.
- 85 Carvalho FA, Barnich N, Sivignon A, *et al.* Crohn's disease adherent-invasive *Escherichia coli* colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. *J Exp Med* 2009;206:2179–89.
- 86 Darfeuille-Michaud A, Boudeau J, Bulois P, *et al.* High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. *Gastroenterology* 2004;127:412–1.
- 87 Rolhion N, Darfeuille-Michaud A. Adherent-invasive *Escherichia coli* in inflammatory bowel disease. *Inflamm Bowel Dis* 2007;13:1277–83.
- 88 Darfeuille-Michaud A, Neut C, Barnich N, *et al.* Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* 1998;115:1405–13.
- 89 Newburg DS, Grave G. Recent advances in human milk glycobiology. *Pediatr Res* 2014;12:24.

Supplemental Material

Materials and methods

Quantifying bacterial invasion

The number of bacteria invading IECs (i) during this one hour incubation was calculated: After the 1 hour inoculation, media were replaced by fresh media containing gentamicin (50 µg/mL); subsequent incubation for 1 hour killed extracellular bacteria that had not invaded. The target IECs were washed with PBS six times, lysed with 1% Triton [W2], and 1:1000 dilutions of lysates plated on Difco agar plates. The number of bacteria that had invaded IECs (i) was quantified as CFUs (Colony Forming Unit) on the agar plates 16 hours after plating.

Quantifying adherent bacteria

To calculate the number of adherent bacteria (a), after 1 hour incubation of ETEC with IECs, cells were washed with PBS thrice and lysed with 1% Triton [W2]. Lysates were diluted 1:2000 and plated on Difco agar plates. After 16 hours of incubation, the total bacteria (t) were quantified as CFU. Adherent bacteria (a) were calculated as: [the number of total bacteria (t)] – [the number of invaded bacteria (i)]:

$$a = t - i$$

Measuring inflammatory response

To measure inflammatory response of IECs to invasion, after 1 hour inoculation of IECs with ETEC, media were replaced by fresh media containing gentamicin (50 µg/mL); the subsequent 1 hour incubation killed extracellular (not having invaded) bacteria. Target IECs were washed with PBS six times, and the cells incubated for 16 hours in fresh antibiotic containing media. Supernatants were stored at -20°C and analyzed for IL-8 by ELISA (Enzyme-Linked ImmunoSorbent Assay).

Quantitative RT-PCR

Total RNAs were extracted with Trizol (Invitrogen, Grand Island, NY). First-strand cDNAs were synthesized by the InVitrogen Superscript III platinum kit following the standard protocol that uses 1 µg of total RNA as the template. Paired primers (supplemental table 1) were designed from a primer bank [W3], and synthesized by Eurofins (Boston, MA); GAPDH, β -actin, and cyclophilin A were internal controls. The relative gene expressions were determined by the delta delta CT method [W4] following triplicate qRT-PCR with SYBR green.

Western blots

T84 cells were washed twice with ice cold PBS, and lysed in lysis buffer containing protease inhibitor cocktail (Cell Signaling, Danvers, MA). Aliquots containing 50 µg of total protein (BCA, Pierce, Rockford, IL) were resolved on SDS/PAGE (Invitrogen, Grand Island, NY) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Signaling proteins were visualized by primary antibodies to CD14 (OriGene Technologies, Rockville, MD), TLR4, MyD88, phosphorylated NF- κ B p65 (Abcam, Cambridge, MA), κ B, phosphorylated Erk, phosphorylated p38, phosphorylated Akt, SOCS1, SOCS2, and SOCS3 (Cell signaling Technology, Danvers, MA), and the secondary antibodies (IRDye 680CW goat-anti mouse or IRDye 800CW goat-anti rabbit) on an Odyssey scanner (Li-cor biotechnology, Lincoln, NE). Area under the curves (arbitrary band absorption units) were normalized to β -actin (Abcam, Cambridge, MA) loading controls, and reported as mean \pm SEM of at least three independent experiments. H4 cells were grown on the bottoms of 96 well plates for 48 hours with 2'-FL. Signaling molecules were measured *in situ*

because of limited analyte; cells were fixed and permeabilized (Fix & Perm; InVitrogen), and stained with the above antibodies and quantified on the Odyssey scanner.

Antibody Arrays

T84 cells (1×10^6 cells) were treated with 2'-FL (2 mg/mL) for 48 hours. After washing with cold PBS, cells were lysed in nondenaturing buffer (Amersham, Piscataway, NJ). Each protein sample (20 μ g) was labeled separately with Cy3 or Cy5 dye and passed through a PD-10 desalting column (Amersham, Piscataway, NJ). The combined labeled protein samples were hybridized to antibody array slides (Clontech Laboratories, Mountain View, CA) for 30 minutes at room temperature. After the series of washes recommended by the manufacturer, the slides were dried and scanned by a ProScanArray HT scanner (PerkinElmer, Waltham, MA), and the data analyzed by ScanArrayGx/ProScanarray software (PerkinElmer, Waltham, MA). Proteins whose expression was significantly altered by the 2'-FL treatment were clustered according to their regulation networks and signaling pathways by Metacore software (GeneGo, <http://trials.genego.com>).

Results

HMOs inhibit ETEC invasion and IL-8 secretion

The classical model for studying signaling responses to ETEC infection is T84 cells [W1]. Cells pretreated for 48 hours with 5 mg/mL HMOs displayed only 12% of the ETEC adherence and 22% of the ETEC invasion of untreated controls (supplemental figure 1A, 1B), with a 60% reduction of IL-8 release (supplemental figure 1C). Pretreatment for only 24 hours attenuated IL-8 secretion 40%, while pretreatment for 1 or 6 hours did not significantly attenuate IL-8 release (supplemental figure 1D).

Increasing the concentration of pretreatment HMOSs to 20 mg/mL for 1 hour also did not attenuate IL-8 secretion (data not shown). Thus, HMOS pretreatment inhibited ETEC infection in a time-dependent manner, and higher concentrations could not compensate for shorter incubation. This time-dependence, in conjunction with the persistence of HMOS inhibition after extensive cell washing, suggested that HMOSs might cause T84 cells to resist infection through direct modulation of an intracellular process.

HMOSs suppress CD14 expression

Candidates for intracellular modulation by HMOS treatment include transcription of CD14, TLR4, MyD88, NF- κ B and IL-8, principal co-receptors and downstream signaling molecules induced by LPS. Of these, the most pronounced attenuation by HMOSs was CD14 mRNA (supplemental figure 2A). ETEC invasion increased CD14 protein expression by ~50%, and HMOS treatment significantly attenuated CD14 protein expression (supplemental figure 2B), measured as lower CD14 protein levels by western blot, and reduced cell-surface CD14 by FACS analysis (supplemental figure 2C). Although CD14 mRNA transcription and protein expression were elevated by ETEC infection, both processes were inhibited by HMOS irrespective of ETEC infection (supplemental figures 2A, B). These data implicated IEC CD14 as a potential target molecule of HMOSs.

To confirm that HMOSs attenuate inflammation through CD14 expression, models were developed in which CD14 was knocked down or overexpressed. CD14 expression in T84 cells, which exhibit high endogenous CD14 expression (supplemental figure 3A), was knocked down by shRNA transfection. Following transfection, CD14 was reduced by ~50% (supplemental figure 3B), and IL-8 induction decreased by ~25% (supplemental figure 3C). This knockdown of CD14 diminished the ability of HMOSs to inhibit the IL-8 induction by ETEC invasion from about 60% to 15% (supplemental figure 3C), demonstrating that HMOS attenuation of inflammation depends upon CD14 expression.

Elevated CD14 induces apoptosis in IECs (not shown). Therefore, CD14 over-expression was performed in HeLa cells, which express low intrinsic levels of CD14 (supplemental figure 3A). CD14 expression was induced by transfection by pCDNA3-huCD14 plasmids, and the transient over-expression confirmed by western blot (supplemental figure 4B). In untreated HeLa cells, the increased IL-8 secretion caused by ETEC infection is presumably mediated by factors other than CD14, consistent with the inability of HMOSs to inhibit this IL-8 induction (supplemental figure 4A). In HeLa cells overexpressing CD14, HMOSs suppressed ETEC-induced IL-8 secretion by ~30% ($P < 0.01$) (supplemental figure 4C). These results are concordant with HMOS quenching of ETEC-induced IL-8 secretion in epithelial cells being strongly dependent upon CD14 expression. Accordingly, CD14 induction, the presumed target of HMOSs, was used to differentiate activities of individual oligosaccharides.

Supplemental figure legends

Supplemental figure 1. T84 response to ETEC is inhibited by HMOSs. T84 were treated with 5 mg/mL HMOSs for 48 hours before exposure to ETEC (MOI 20). A) Adherence was reduced to 12%. **B)** ETEC invasion was reduced to 22% **C)** IL-8 secretion was reduced to 40%. **D)** IL-8 reduction was time dependent. Means \pm SEM, $n=12$. *, $p \leq 0.05$; **, $p \leq 0.01$ by ANOVA.

Supplemental figure 2. CD14 expression in ETEC-infected T84 cells is inhibited by HMOSs. T84 were treated with 5 mg/mL HMOS for 48 hours before exposure to ETEC (MOI 20). A) HMOSs attenuate CD14 mRNA and IL-8 mRNA induction by ETEC infection, $n=3$. **B)** HMOSs inhibit CD14 expression, $n=3$. **C)** cell surface CD14 is reduced by HMOSs, $n=3$. . *, $p \leq 0.05$; **, $p \leq 0.01$ by ANOVA.

Supplemental figure 3. T84 expression of CD14 is sensitive to knock-down and HMOSs. T84 and shRNA
T84 cells were treated with 5 mg/mL HMOSs for 48 hours before exposure to ETEC (MOI 20). A) CD14 expression levels among IECs, n=3 **B)** CD14 expression in pRS-shCD14 RNA transfected T84 cells; the pRS vector and scrambled pRS-RNA are negative controls, n=3. **C)** IL-8 secretion induced by ETEC invasion of T84 cells is attenuated by CD14 knockdown, **and** by HMOSs in controls; residual IL-8 in CD14 knocked-down T84 cells is unchanged by HMOS treatment, consistent with hMOS inhibition being specific for CD14 mediated IL-8 induction, n=6. **, $p \leq 0.01$; ***, $p \leq 0.001$ by ANOVA.

Supplemental figure 4. Overexpression of CD14 increases the IL-8 response to ETEC infection, which is attenuated by HMOS. HeLa cells, intrinsically low in CD14, when transfected with the gene for CD14, overexpressed CD14 without inducing toxicity. **A)** Prior to transfection, HeLa cell IL-8 induction by ETEC infection is not sensitive to HMOS treatment (n=6). **B)** HeLa cells transfected by a CD14 plasmid construct overexpress CD14 in proportion to the plasmid dose (n=3). **C)** IL-8 induction by ETEC infection in CD14 overexpressing HeLa cells is inhibited by HMOS, consistent with HMOS inhibition of IL-8 being mediated through CD14 (n=6). IL-8 data (A&C) normalized to untreated ETEC infection (100%). ** indicates significant difference between mean values, $p \leq 0.01$.

Supplemental figure 5. IL-8 response to 1-100,000 ng/mL LPS in T84 cells. T84 IECs respond at 10 μ g/mL, and a dose of 100 μ g/mL (~IC50) was used for T84 cell stimulation in these studies.

Supplemental figure 6. Signaling mediators of the MIF pathway whose expression is altered by 2'-FL.
T84 cells were treated with 2'-FL for 48 hours, the proteins extracted, and applied to a signaling

molecule antibody array. Meta core analysis identified the MIF network signaling pathways whose intermediates are most effected by 2'-FL. Genes whose expression is significantly induced by 2'-FL treatment are labeled red, and include IP3 receptor, MMP-1 and Stromelysin-1. Genes whose expression is significantly repressed by 2'-FL treatment are labeled green, and include the cytokines IFN- γ , IL-8, IL-6, IL-1 β , TNF- α , IL-1 α and IL-2.

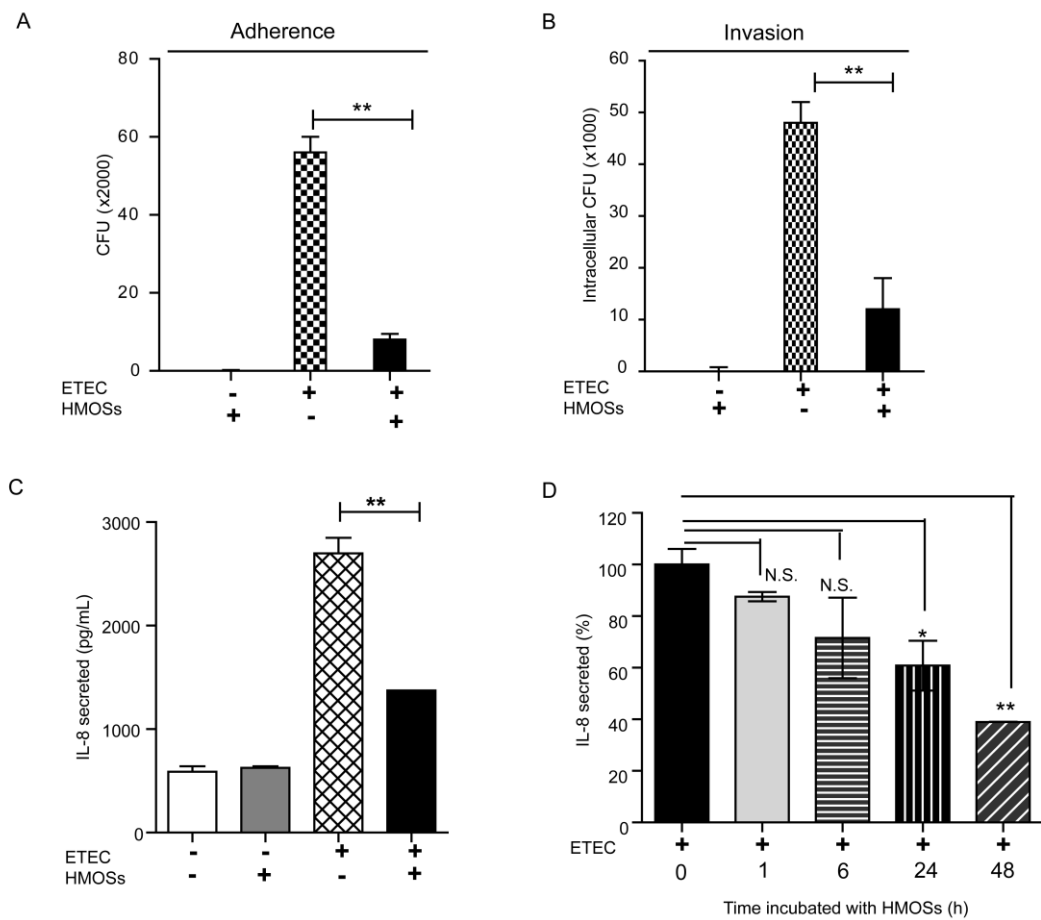
Supplemental figure 7. Comparison of H&E stained colon sections from AIEC infected mice with or without 2'-FL pretreatment. Images are representative of six mice each of PBS controls, AIEC infected, and 2'-FL pretreated prior to AIEC inoculation. Red arrows indicate inflammatory cell infiltrates and epithelial cell sloughing induced by AIEC infection, which are not seen in colons from 2'-FL pretreated mice or saline controls. Bars = 50 μ M.

WEB REFERENCES

- W1. Crane JK, Azar SS, Stam A, Newburg DS. Oligosaccharides from human milk block binding and activity of the Escherichia coli heat-stable enterotoxin (STa) in T84 intestinal cells. J Nutr 1994;**124**:2358-64.
- W2. Kawada M, Chen CC, Arihiro A, Nagatani K, Watanabe T, Mizoguchi E. Chitinase 3-like-1 enhances bacterial adhesion to colonic epithelial cells through the interaction with bacterial chitin-binding protein. Lab Invest 2008;**88**:883-95.
- W3. Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res 2003;**31**:e154.
- W4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;**25**:402-8.

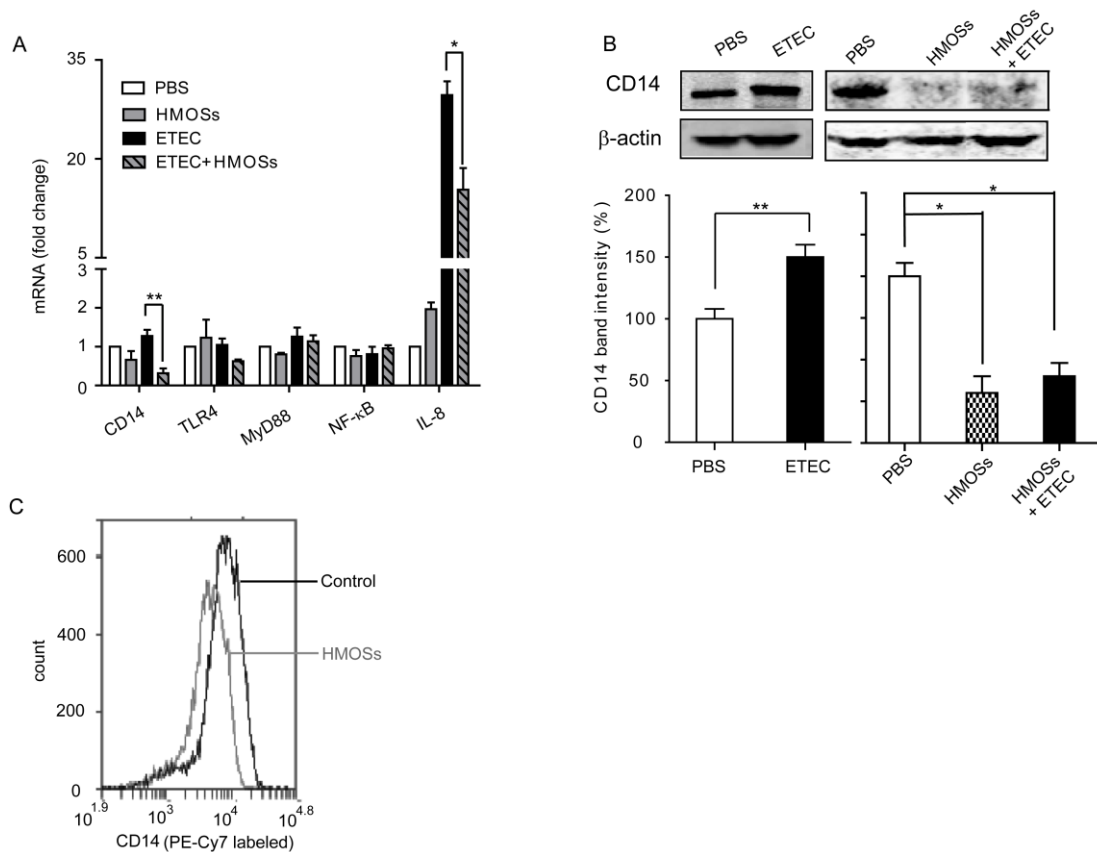
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Supplemental figure 1



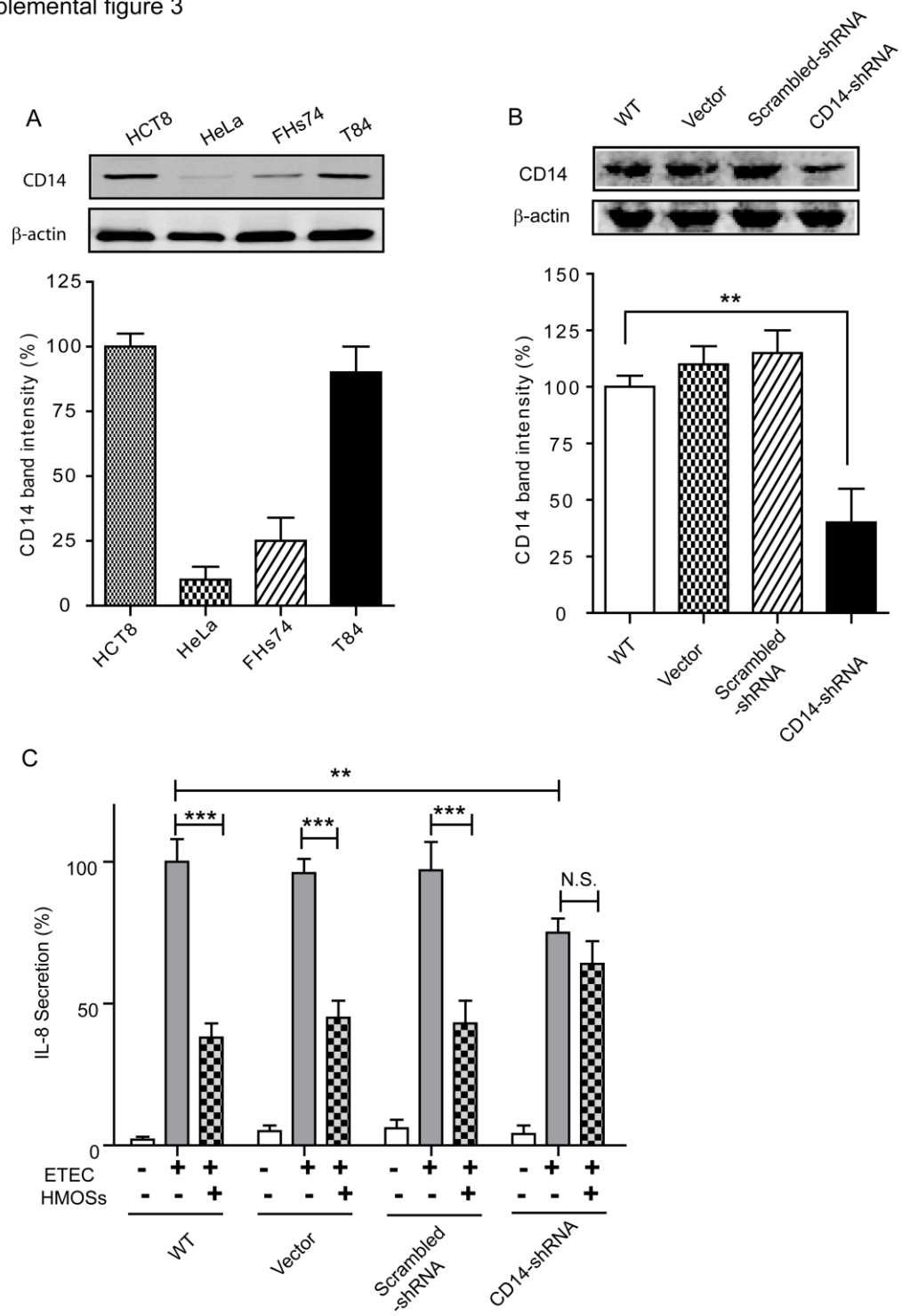
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Supplemental figure 2

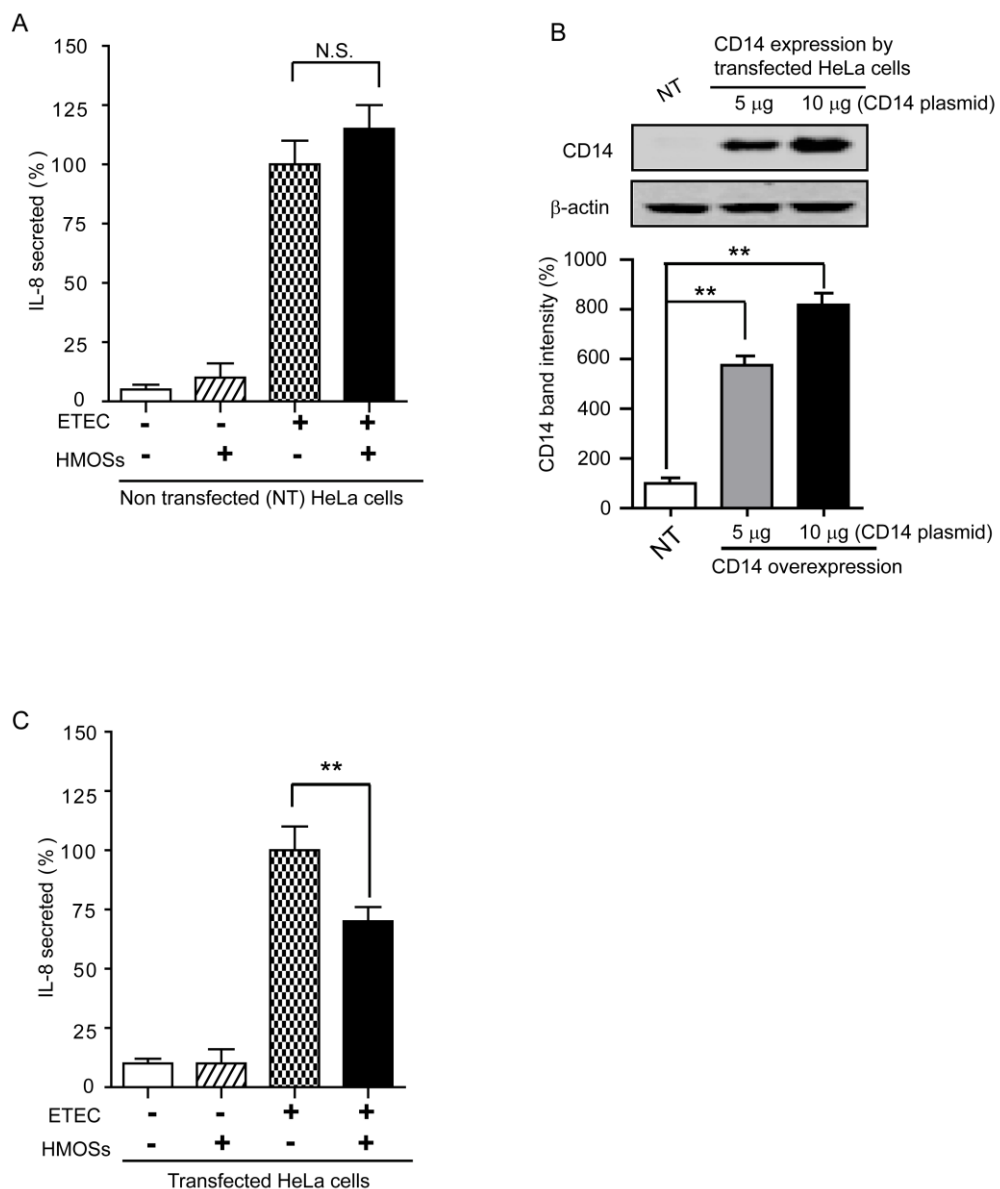


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Supplemental figure 3

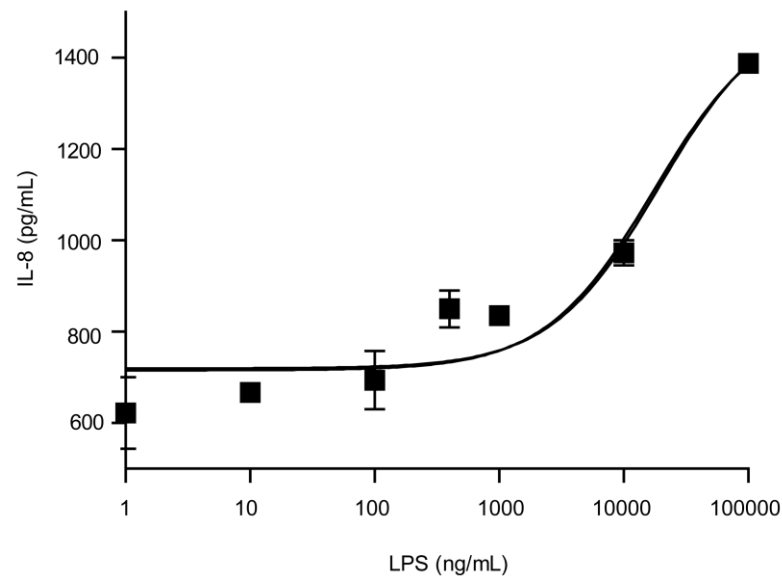


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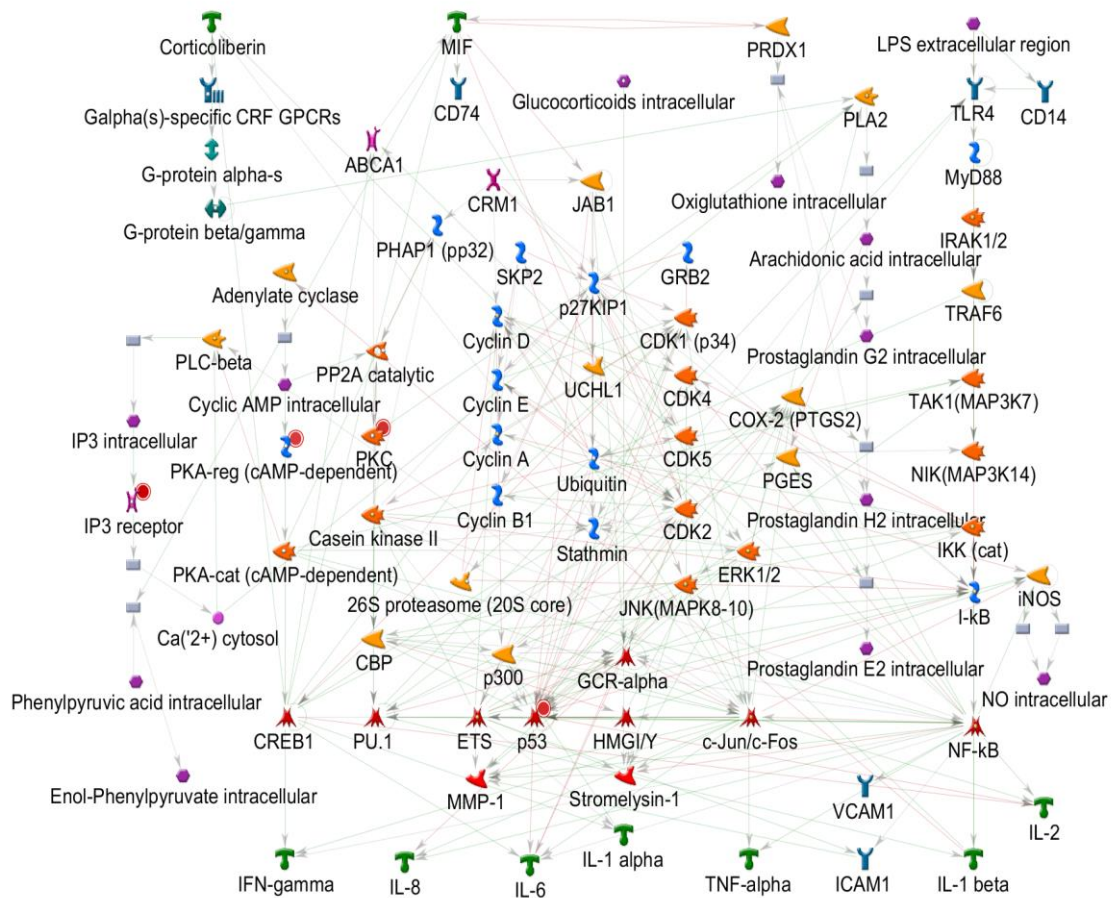
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Supplemental figure 5



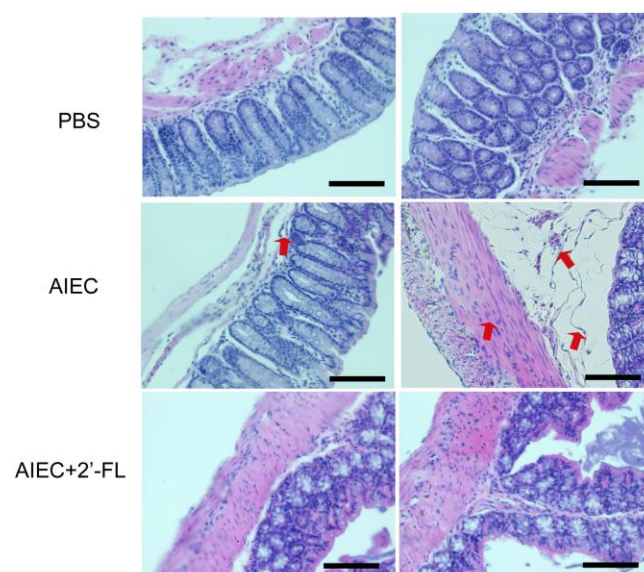
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Supplemental figure 6



Supplemental figure 7. Comparison of H&E stained colon sections from AIEC infected mice with or without 2'-FL pretreatment. Images are representative of six mice each of PBS controls, AIEC infected, and 2'-FL pretreated prior to AIEC inoculation. Red arrows indicate inflammatory cell infiltrates and epithelial cell sloughing induced by AIEC infection, which are not seen in colons from 2'-FL pretreated mice or saline controls. Bars= 50 μ M.

Supplemental figure 7



Supplemental table 1. Primers used in qRT-PCR .

Gene	Primers	Primer Sequence	Amplicon (bp)
CD14	FW	ACGCCAGAACCTTGTGAGC	122
	REV	GCATGGATCTCCACCTCTACTG	
TLR4	FW	GTACCTGGGGAACAACCTCTT	146
	REV	GCAGCTTGACTAGACTCTCCA	
MyD88	FW	ATCGCTGTTCTTGAACCCTCG	199
	REV	CTCACGGTCTAACAAGGCCAG	
NF- κ B	FW	AATTAACGGCGACAATCTGGAA	205
	REV	ACTTCACAAGCATAGCCATCAG	
IL-8	FW	TTTTGCCAAGGAGTGCTAAAGA	194
	REV	AACCCTCTGCACCCAGTTTTC	
GADPH	FW	TGTTGCCATCAATGACCCCTT	202
	REV	CTCCACGACGTACTCAGCG	
Actin	FW	CATGTACGTTGCTATCCAGGC	250
	REV	CTCCTTAATGTCACGCACGAT	