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 g/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 g/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 g of total RNA as the template. Paired primers (supplemental table 1) were designed from a primer bank [W3], and synthesized by Eurofins (Boston, MA); GADPH, -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 g 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-B p65 (Abcam, Cambridge, MA), iB, 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 -actin (Abcam, Cambridge, MA) loading controls, and reported as mean ± 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 □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

HMOSs 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 HMOSs 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-□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 ± SEM, n=12. *, p≤0.05; **, p≤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≤0.05; **, p≤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≤0.01; ***, p≤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≤0.01.

Supplemental figure 5. IL-8 response to 1-100,000 ng/mL LPS in T84 cells. T84 IECs respond at 10 □g/mL, and a dose of 100 □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-□, IL-8, IL-6, IL-1□, TNF-□, IL-1□ 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 □M.

WEB REFERENCES

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