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MAJOR ARTICLE

Human Milk Oligosaccharides Protect Bladder Epithelial Cells Against Uropathogenic *Escherichia coli* Invasion and Cytotoxicity

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The invasive pathogen uropathogenic *Escherichia coli* (UPEC) is the primary cause of urinary tract infections (UTIs). Recurrent infection that can progress to life-threatening renal failure has remained as a serious global health concern in infants. UPEC adheres to and invades bladder epithelial cells to establish infection. Studies have detected the presence of human milk oligosaccharides (HMOs) in urine of breast-fed, but not formula-fed, neonates. We investigated the mechanisms HMOs deploy to elicit protection in human bladder epithelial cells infected with UPEC CFT073, a prototypic urosepsis-associated strain. We found a significant reduction in UPEC internalization into HMO-pretreated epithelial cells without observing any significant effect in UPEC binding to these cells. This event coincides with a rapid decrease in host cell cytotoxicity, recognized by LIVE/DEAD staining and cell detachment, but independent of caspase-mediated or mitochondrial-mediated programmed cell death pathways. Further investigation revealed HMOs, and particularly the sialic acid-containing fraction, reduced UPEC-mediated MAPK and NF-κB activation. Collectively, our results indicate that HMOs can protect bladder epithelial cells from deleterious cytotoxic and proinflammatory effects of UPEC infection, and may be one contributing mechanism underlying the epidemiological evidence of reduced UTI incidence in breast-fed infants.

Keywords. uropathogenic *E. coli*; human milk oligosaccharides; urinary tract infection; bladder epithelial cells; apoptosis; cell adhesion.

Uropathogenic *Escherichia coli* (UPEC) is the leading cause of urinary tract infections (UTIs) in healthy adults, accounting for >100 million cases annually worldwide [1, 2]. More than 1 million children are affected by UTIs each year, and remain susceptible to recurrent infections, putting them at risk of severe renal damage and increased mortality [3]. UPEC infections typically begin in the bladder (cystitis), with the potential to ascend to the kidney via the ureters, resulting in pyelonephritis, and in severe cases chronic renal failure or bacteremia/sepsis [4, 5]. Despite the high prevalence of UTIs, an effective strategy to prevent UPEC infection in neonates has yet to be realized.

To establish infection, UPEC must adhere to and invade bladder epithelium. Upon invasion, UPEC alters multiple signaling cascades and disrupts the localization of many subcellular elements, triggering severe damage to bladder epithelial cells. Cytotoxicity is characterized by apoptosis and rapid exfoliation of the superficial cell layer lining the surface of the bladder lumen [6]. Although shedding of dead cells may promote clearance of entrapped bacteria from the infected bladder tissue, the loss of epithelial integrity can also allow bacterial dissemination into deeper tissues [7].

Emerging evidence suggested that breast-fed infants are at significantly lower risk of acquiring infections [8–10]. It is postulated this protection may in part derive

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from human milk oligosaccharides (HMOs), complex mixtures of carbohydrates present in high abundance in human milk (10–15 g/L) but absent in formula milk [11–13]. The HMO backbone contains lactose at the reducing end, is elongated with alternating units of N-acetylglucosamine and galactose, and can be fucosylated. In addition to these neutral (nonsialylated) oligosaccharides, some HMOs also contain sialylated oligosaccharides with 1 or more sialic acid (in humans exclusively N-acetylneuraminic acid, Neu5Ac) [13]. Due to the many different combinations of elongation, fucosylation, and sialylation, human milk contains a diverse array of highly complex oligosaccharides. Each woman synthesizes and secretes a unique HMO composition profile [13]. HMOs serve as soluble decoy receptors for surface adhesins of various microbes, and may competitively interfere with pathogen attachment to host cellular receptors, impeding colonization or infection. This has been demonstrated for *Entamoeba histolytica*, *Streptococcus pneumoniae*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, *Campylobacter jejuni*, *and Helicobacter pylori*, as well as viruses [14–20].

HMOs are absorbed in the infant's intestine and have been detected in the urine of breast-fed, but not formula-fed, infants [21, 22]. A series of smaller clinical studies have found term and preterm infants to be 2–3 times less likely to develop UTIs than formula-fed controls [8–10]. These observations raise the question whether HMOs contribute to urological health in breast-fed infants. Therefore, we investigated whether HMOs confer protection against UPEC strain CFT073, a prototypic agent of urosepsis previously obtained as a blood isolate from a patient with pyelonephritis [23].

METHODS

Bacterial Strain, Cells, Media, and Growth Conditions

Wild-type UPEC strain CFT073 (O6:K2:H1; ATCC 700928) was grown to stationary phase at 37°C in Luria-Bertani broth prior to infection. Human bladder epithelial cells (5637 ATCC HTB-9) were cultured in RPMI 1640 (Invitrogen) media supplemented with 10% heat-inactivated fetal bovine serum at 37°C in humidified air with 5% CO_2.

Treatment of Bladder Epithelial Cells With HMOs

HMOs (pooled, neutral and acidic), galactooligosaccharides (GOSs), or 3′SL fractions were prepared in serum-free RPMI and filter-sterilized with a 0.22-μM PVDF filter (Whatman). HTB-9 monolayers were treated with prewarmed HMOs at 5, 10, or 15 mg/mL for at least 16 hours unless stated otherwise; untreated cells served as a control. Oligosaccharides were also maintained in the cell media during the initial 2 hours of UPEC infection (designated as the adhesion time point) but washed out for the gentamicin treatment phase (designated as the invasion time point). More detailed methods can be found in the Supplementary Data.

RESULTS

HMOs Interfere With UPEC Entry Into Human Bladder Epithelial Cells

Oligosaccharides can interact directly with the surface of bacteria, with the potential to inhibit attachment to host cells [14–17]. To examine whether HMOs affect UPEC adhesion to human bladder epithelial cells, we infected HTB-9 cell monolayers with UPEC in the presence or absence of 15 mg/mL of HMOs isolated from pooled human milk. Based on prior studies, this HMO concentration is optimal for determining biological effects in intestinal epithelial cell–based assays [24]. This was also confirmed by a dosage and time-dependent assay (Supplementary Figure 1*A*). HTB-9 cells were preconditioned with HMOs in serum-free RPMI for at least 16 hours prior to infection with UPEC. Serum-free media was used to assess the effect of HMOs without possible interference caused by the serum. We observed a modest, but statistically insignificant, decrease in UPEC attachment in HMO-treated cells compared to untreated cells (Figure 1*A*). In contrast, we discovered that the level of UPEC uptake ("invasion") into HTB-9 cells was markedly diminished in cells previously incubated with HMOs (Figure 1*A*). Whereas approximately 10% of the initial UPEC inoculum entered HTB-9 cells in the absence of HMOs, only approximately 1.7% of inoculated UPEC was able to penetrate into HMO-pretreated HTB-9 cells ($P < .001$). In these assays, we observed no detectable difference in bacteria recovered from the supernatant in the absence or presence of HMOs (Figure 1*B*), consistent with identical UPEC growth curves monitored in the presence or absence of HMOs (data not shown). Thus, inhibition of UPEC invasion is not an indirect consequence of bacterial growth suppression or killing.

HMOs Reduce UPEC-Mediated Cytotoxicity and Lifting of Human Bladder Epithelial Cells

UPEC infection has been reported to induce apoptotic cell death in bladder epithelium [25]. To gain insight into whether HMOs protect host cells against UPEC cytotoxicity, we assayed cell death via LIVE/DEAD viability assay coupled with fluorescent microscopy at the above-mentioned adherence and invasion time points. UPEC infection produced high levels of cell death in HTB-9 cells as evidenced by abundant ethidium homodimer-1 staining (Figure 2*A*); similar cell injury was not evident in cells incubated with UPEC supernatant or heat-killed UPEC (Supplementary Figure 1*B*), indicating that both viable UPEC and direct cell contact are required to induce cell death. In contrast to untreated cells (approximately 300 dead cells per field of view), cells pretreated with HMOs withstood UPEC cytotoxicity significantly better during the adherence period (approximately 70 dead cells per field of view) (Figure 2*A*, Supplementary Figure 2*A*). Similar observations were made at the invasion time point (after 2 hours of gentamicin treatment),

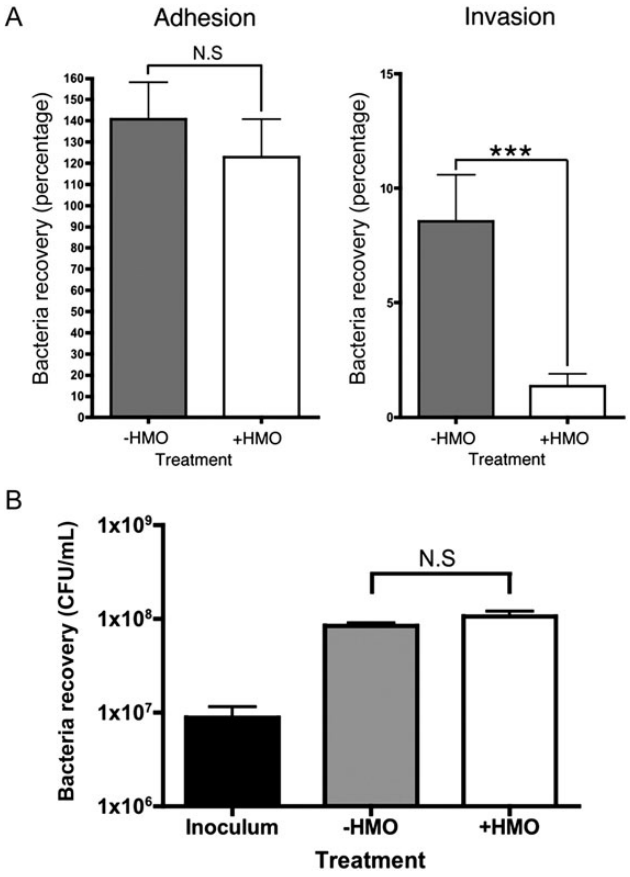

Figure 1. Uropathogenic *Escherichia coli* (UPEC) adhesion and invasion in human kidney epithelial cells pretreated with human milk oligosaccharides (HMOs). *A*, Confluent human kidney epithelial cells (HTB-9) were pretreated with 15 mg/mL of HMOs for 16 hours prior to infection with UPEC CFT073. The level of initial bacteria attachment was assessed followed by 2 hours of infection (left). Cells were then washed twice with phosphate-buffered saline and incubated with 100 μg/mL of gentamicin for 2 hours before being harvested for colony-forming unit (CFU) counts to assess level of invasion (right). Percentage adhesion and invasion were obtained by dividing bacteria recovered at specific time point by the average of initial inoculum. Results are mean ± SEM, n = 3 from 5 independent experiments. N.S., not significant. ***$P < .001$ as determined by Student unpaired 2-tailed *t* test. *B*, Bacteria recovered after 2 hours of incubation in serum-free RPMI in the absence of HMOs (–HMO) or 15 mg/mL of HMOs (+HMO).

where the protective effect of HMOs is also reflected in a higher level of viability (approximately 500 cells per field of view) relative to untreated cells (<5 viable cells per field of view) (Figure 2*A*, Supplementary 2*A*).

UPEC are known to cause severe exfoliation of the mucosal layer of bladder epithelium [6]. Concurrent to the differences in cell viability observed between untreated and HMO-treated cells, we also noted a dramatic difference in cell attachment between these 2 populations. UPEC produced rapid detachment of HTB-9 cells at the adherence and invasion time points (approximately 1.5% of viable cells attached on coverslips); in contrast, cells pretreated with HMOs remained largely intact (approximately 90% viable cells) (Figure 2). Further analysis over a concentration range of 5 mg/mL, 10 mg/mL, and 15 mg/mL of HMOs revealed dose dependency in the protection against UPEC-mediated bladder epithelial cytotoxicity (LIVE/DEAD staining) and cell lifting (Figure 2*C* and 2*D*; Supplementary Figure 2*B*).

HMOs Promote Bladder Epithelial Cell Structural Integrity and Cell–Cell Adhesion During UPEC Infection

Cell-to-cell adhesion within epithelial layers is governed by a broad collection of cytoskeletal and signaling proteins, including focal adhesion complex and various specialized intercellular junction proteins [26, 27]. Failure to properly coordinate expression and assembly of these proteins not only weakens cell integrity and compromises cellular adhesion function, but also disrupts downstream intracellular signaling [27].

Recently, it was revealed that UPEC UTI89 targets paxillin, a 68-kDa focal adhesion molecule, for degradation in HTB-9 cells [28]. Consistent with this finding, we documented a significant loss of paxillin protein in HTB-9 cells at the invasion time point (Figure 3*A*). Remarkably, preincubation with HMOs inhibited degradation of paxillin by UPEC as measured by Western blot analysis (Figure 3*A*). To achieve a more comprehensive analysis of HMO effects on focal adhesion complexes, we examined the abundance of other paxillin-associated adhesion molecules. Specifically, we studied β1-integrin, a surface integral protein that facilitates linkage between extracellular matrix to focal adhesion molecules for cell anchorage, as well as focal adhesion kinase (FAK) and vinculin, which are responsible for triggering downstream signaling cascades and maintaining proper structural organization. Similar to paxillin, a strong degradation of β1-integrin was found in UPEC-infected cells, and HMOs were able to inhibit this process (Figure 3*B*). On the other hand, even with overt cytotoxicity, expression levels of both FAK and vinculin appeared to be unaltered during UPEC infection (Figure 3*A* and 3*B*), despite a recent study showing that FAK is indispensable for active UPEC invasion into epithelial cells [29]. Our results suggest that HMOs provide a synergistic effect to prohibit selective focal adhesion complex components from UPEC-mediated degradation, helping explain the ability of HMOs to prevent cell detachment (Figure 3*B*).

To further tease out the role of HMOs in preserving cellular adhesion molecules during UPEC infection, we expanded our study to investigate the status of a group of intercellular junction proteins. Epithelial cadherin (E-cadherin), a member of cadherin junctions responsible for cell adhesion, was significantly reduced in UPEC-infected cells. Interestingly, HMOs did not prevent degradation of E-cadherin as previously seen with paxillin and β1-integrin (Figure 3*C*). Desmocollin 2/3

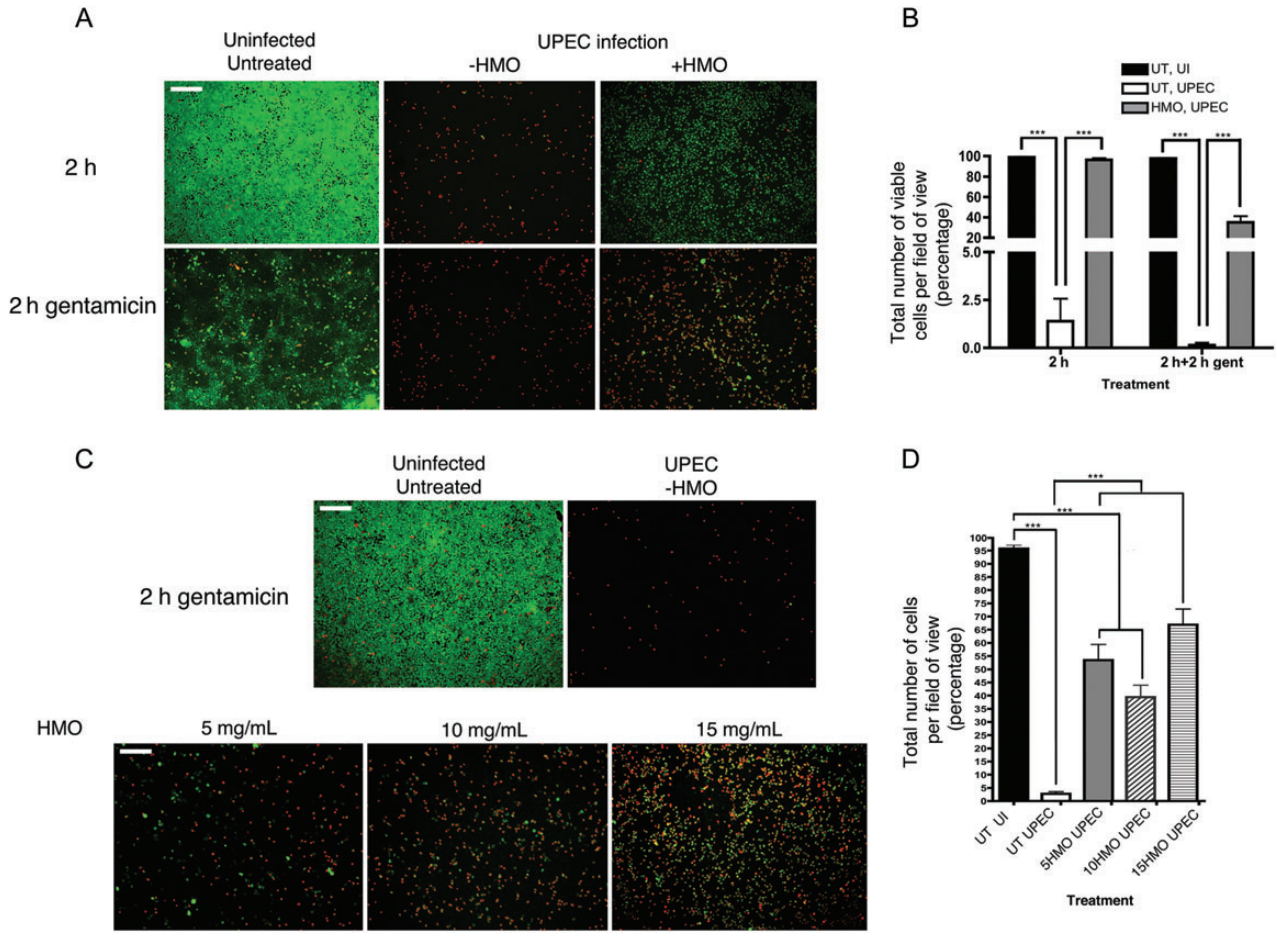

Figure 2. Human milk oligosaccharides (HMOs) facilitate dose-dependent protection from uropathogenic *Escherichia coli* (UPEC)–induced cell death and detachment of bladder epithelial cells. *A*, Representative fluorescent images of untreated or HMO-pretreated HTB-9 cells stained with LIVE/DEAD cell viability/cytotoxicity kit for mammalian cells (green = live cells, red = dead cells). Cells either remained uninfected or infected with UPEC for 2 hours (top panel) or washed twice with phosphate-buffered saline and incubated in serum-free RPMI supplemented with gentamicin (bottom panel). Scale bar = 200 μm. *B*, Quantitative analysis of viable HTB-9 cells infected with UPEC with or without HMOs pretreatment. The percentage of viability is obtained by dividing the numbers of viable cells by the total number of attached cells counted per field of view. All cell counts were averaged from multiple fields of view (n > 3 per sample). Experiments were performed in duplicate and repeated at least 3 times. *C*, HMOs protect against cell death in a dose-dependent manner. Representative fluorescent images illustrating viability of UPEC-infected HTB-9 cells in response to different dosages of HMOs pretreatment. HTB-9 cells pretreated with HMOs for 16 hours at indicated concentrations (5, 10, and 15 mg/mL) followed by 2 hours of infection with UPEC and an additional 2 hours of gentamicin treatment as previously described. Scale bar = 200 μm. *D*, Quantitative analysis of viable HTB-9 cells that are uninfected, untreated (UI, UT), or infected with UPEC without (UT) or with HMOs pretreatment at different concentrations (5, 10, 15 mg/mL). The percentage of viability is obtained by dividing the numbers of viable cells by the total number of attached cells counted per field of view. All cell counts were averaged from multiple fields of view (n > 3 per sample). Experiments were performed in duplicate and repeated at least 3 times. ***$P < .001$, determined by 1-way analysis of variance (ANOVA) nonparametric test followed by Dunnett post test.

(Dsc2/3) belongs to a second group of cadherin superfamily called desmosomes, which are intercellular junctions that confer structural strength to epithelial cells. We found expression of Dsc2/3 isoforms was suppressed by UPEC during the invasion process, but this reduction was blocked by HMOs pretreatment (Figure 3*D*). We immunolocalized Dsc2/3 in infected HTB-9 cells and found that normal Dsc2/3 localization to the cell periphery was disrupted by UPEC. This is evident by the loss of Dsc2/3 localization at the cell periphery in cells with invading bacteria; cells without invading bacteria retained proper Dsc2/3 localization (Figure 3*E*), suggesting that UPEC alters Dsc2/3 organization during the invasion process. When preexposed to HMOs, UPEC-invaded cells did not suffer the extensive morphological changes in Dsc2/3 localization seen with untreated cells (Figure 3*E*). Thus, HMO-mediated resistance in cell lifting caused by UPEC could be a consequence of reduced bacterial invasion and protection against degradation of components important for structural and adhesion properties.

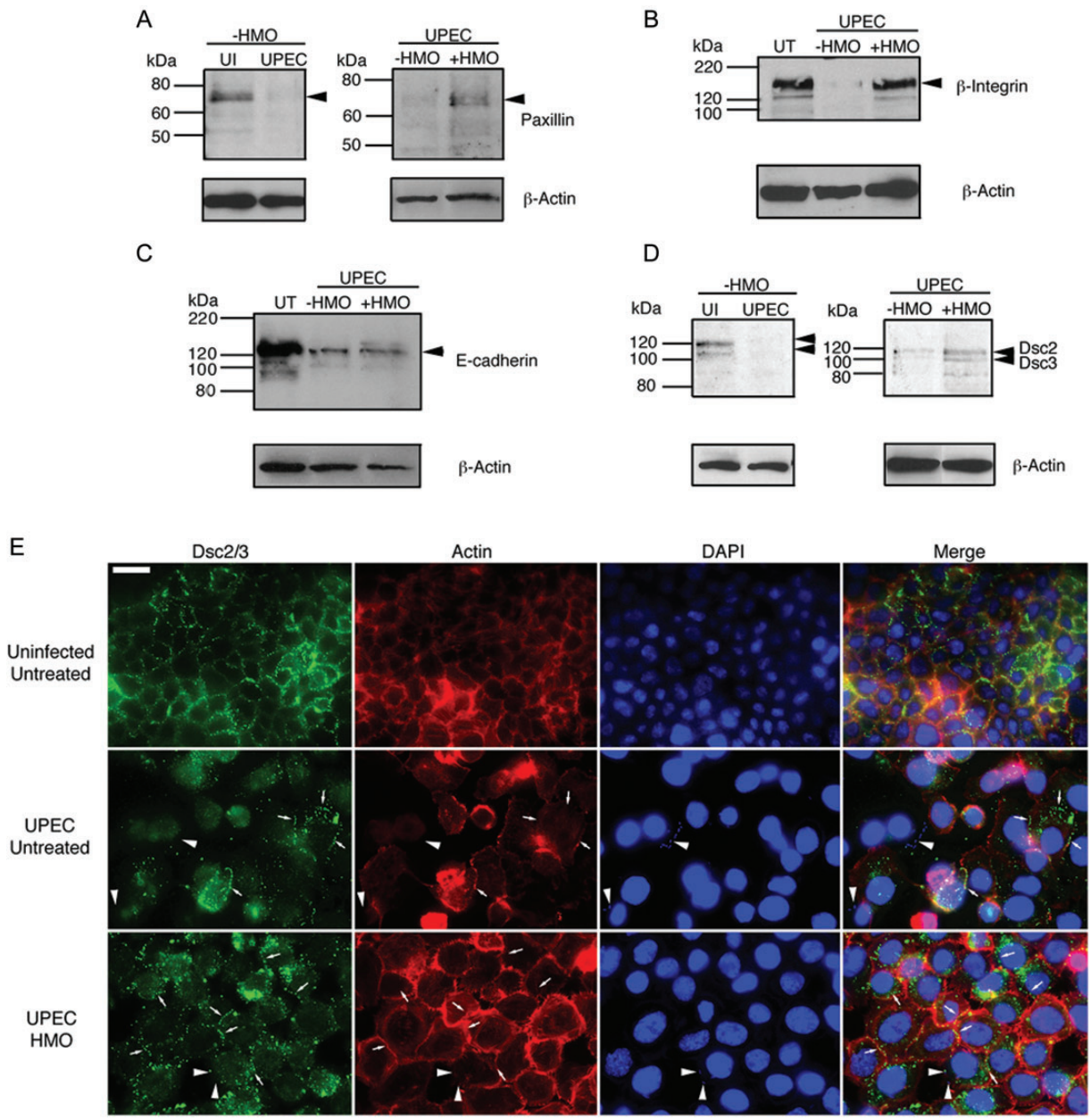

Figure 3. Human milk oligosaccharides (HMOs) block uropathogenic *Escherichia coli* (UPEC)–mediated degradation of cell adhesion molecules. *A-D*, Untreated (UT) or HMO-treated HTB-9 cells were infected with UPEC for 2 hours at a multiplicity of infection (MOI) of 5–20 followed by 2 hours of gentamicin treatment (100 μg/mL) and then lysed for immunoblot analysis. Immunoblots illustrating protein abundance of focal adhesion protein paxillin (*A*), β1-integrin (*B*), E-cadherin (*C*), desmocollin 2/3 (Dsc2/3; *D*). Actin was used as loading control. Blots are representative of at least 2 experimental repeats. *E*, Immunolocalization of desmocollin 2/3 (Alexa 488, green) and actin (treated with phalloidin 594, red) in uninfected (UI) cells and UPEC-infected cells with HMOs (+HMO) or without HMOs (–HMO) pretreatment. Cells were infected with UPEC at an MOI of 1–3 for 2 hours followed by 2 hours of gentamicin treatment to remove unbound and extracellular bacteria. Arrowheads indicate localization of internalized bacteria, and arrows indicate Dsc2/3 localization. Scale bar = 20um.

HMO Protection Against Bladder Epithelial Cytotoxicity Is Both Caspase and Cytochrome C Independent

Programmed cell death, or apoptosis, is a multifaceted process regulated by diverse signal transduction pathways. Among the central apoptosis regulators are cysteine proteases caspase-3 and caspase-7, which mediate protein cleavage events to activate cell death. UPEC-induced apoptosis has been linked to caspase 3 activation [28, 30]. To test whether HMOs interfere with this process, we measured total caspase 3 levels; the caspase cleavage pattern and activation were indistinguishable between untreated and HMO-treated cells (Supplementary Figure 3*C* and 3*D*), suggesting that HMOs protect against bladder epithelial cytotoxicity

in a caspase-independent manner. We also examined caspase-independent programmed cell death involving apoptosis-inducing factor (AIF) and cytochrome C, which are released into the cytoplasm by mitochondria in response to death signals [31]. Neither UPEC infection nor HMOs showed any influence in the release of AIF or cytochrome C (Supplementary Figure 3*E* and 3*F*), eliminating the possibility that HMOs block cytotoxicity though inhibition of mitochondria-mediated death signaling.

HMOs Suppress UPEC-Mediated MAPK and NF-κB Activation

Responses generated by focal adhesion complexes have been correlated to the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) cascades [32, 33], which dictate a wide range of physiological events, including cell death, survival, proliferation, adhesion, and structural integrity [34–37]. Numerous bacteria exploit these signaling networks for their own survival benefit within the host [38–40]. For example, pyelonephritis-associated UPEC have been reported to activate MAPK family members, triggering apoptosis of bladder and renal epithelial cells [30, 41–43], whereas *Clostridium difficile* stimulates NF-κB activation, leading to apoptosis, junction degradation, and cell detachment within the intestinal epithelial monolayer [39].

The MAPK family is comprised of 3 major kinases: the extracellular signal-regulated kinase (ERK), the c-Jun NH2-terminal kinase (JNK), and the p38-MAP kinase [34]. We corroborated a dramatic augmentation of phosphorylated MAPK proteins in infected HTB-9 cells, including phospho-p38 (Figure 4*A*) and phospho-pErk1/2 (Figure 4*B*), but not phospho-JNK (data not shown), during UPEC infection. These activation events were completely inhibited by pretreatment of cells with HMOs (Figure 4*A* and 4*B*).

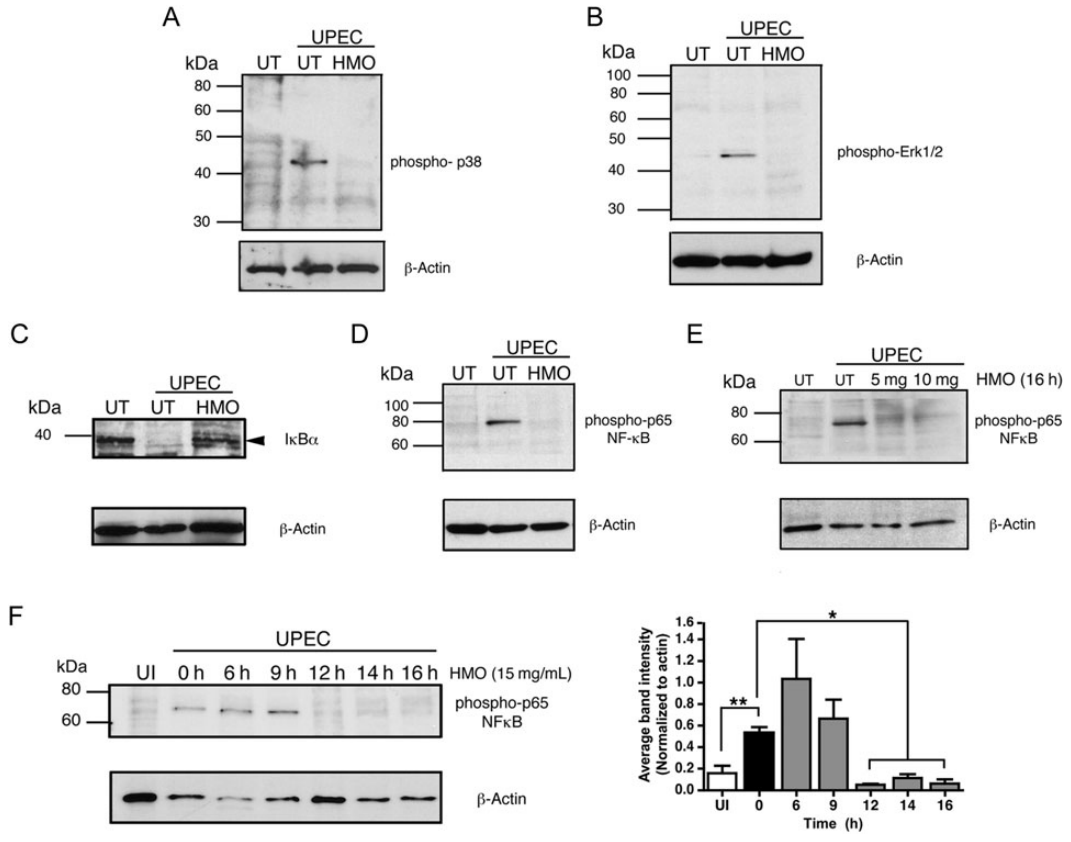

Figure 4. Human milk oligosaccharides (HMOs) suppress uropathogenic *Escherichia coli* (UPEC)–mediated mitogen-activated phosphorylation kinase (MAPK) signaling. HTB-9 cells were either untreated (UT) or pretreated with HMOs prior to UPEC CFT073 infection. Whole cell lysates were solubilized in RIPA lysis buffer after 2 hours of infection followed by 2 hours of gentamicin treatment for immunoblotting for phosphorylated-MAPK signaling molecules. Immunoblot of phospho-p38 MAPK (38 kDa) (*A*) and phosphor-Erk1/2 (42, 44 kDa) (*B*) with or without 16 hours of 15 mg/mL of HMO pretreatment. Immunoblot depicting levels of IkBα (39 kDa) (*C*) and phospho-p65 NF-κB (65 kDa) (*D*) in UPEC-infected HTB-9 cells with or without 16 hours of HMO pretreatment. *E*, Immunoblot of phospho-p65 in HTB-9 cells pretreated with HMOs for 16 hours at 5, 10, and 15 mg/mL. *F*, Immunoblot of phospho-p65 in HTB-9 cells pretreated with 15 mg/mL of HMOs for 0, 6, 9, 12, 14, and 16 hours prior to UPEC infections. UI indicates uninfected, untreated cells. Actin was used as loading control. Bar graph illustrates the relative expression levels of phospho-p65 evaluated from 3 independent immunoblot assays (n = 3); band intensities were quantified and normalized using Image J. $^{*}P < .05$, $^{**}P < .01$, determined by unpaired Student *t* test.

NF-κB is negatively regulated by IκB. In response to external stimuli, IκBα becomes proteasomally degraded, enabling phosphorylation of NF-κB to activate downstream signals. We examined levels of both IκBα along with phospho-p65 NF-κB under the influence of HMOs during UPEC invasion, and found that expression of IκBα was markedly diminished (Figure 4*C*), coinciding with a strong upregulation of phospho-NF-κB p65 (the active form) in UPEC-treated HTB-9 cells (Figure 4*D*). In contrast, cells preconditioned with HMOs accumulated IκBα and exhibited undetectable levels of phospho-p65 NF-kB (Figure 4*C* and 4*D*). Gradual decreases in phospho-p65 occurred as HMOs increased from 5 mg/mL to 15 mg/mL (Figure 4*D* and 4*E*), suggesting the decreased cell death correlates with p65 NF-κB activation (Figure 4*D*). A kinetic analysis also revealed cells treated for >12 hours displayed a significant reduction in phospho-p65 NF-kB compared to untreated cells at time 0 ($P < .05$; Figure 4*F*). This suggests that increased concentration and extended duration of HMO treatment increase the effect of dampening UPEC-induced inflammatory activation and cell death.

The Sialic Acid–Containing Fraction of HMOs Is Essential in Blocking UPEC-Mediated NF-κB Activation

HMOs are a mixture of structurally diverse and complex molecules comprising neutral (nonsialylated) and acidic (sialylated) oligosaccharides (Figure 5*A*). In comparison to HMOs, GOSs, found in some infant formula, consist of linear chains of 2–6 galactose residues linking to a single glucose (Figure 5*A*); notably, GOSs do not contain sialic acid. Because oligosaccharides have been proposed to block bacteria–host cell interaction, we assessed whether the same effect can be recapitulated with GOS treatment. Cells preexposed to 15 mg/mL of GOS were significantly less susceptible to UPEC invasion, by approximately 80% compared to untreated cells (Figure 5*B*). However, through LIVE/DEAD staining, we found that cells treated with GOSs exhibited high levels of cell death and shedding during infections, resembling untreated cells (Figure 5*C*). Cell proliferation assays also revealed that GOSs consistently failed to preserve cell viability at the same level as HMOs (Figure 5*D*). These results suggested that although GOSs have the capacity to block UPEC invasion, they are unable to prevent cell death and lifting in a similar manner to HMOs. Finally, corroborating our hypothesis that HMO protection against cell death and detachment correlates to inhibition of NF-κB and MAPK activation, we found that GOSs neither prevented p38 nor p65 NF-κB phosphorylation in response to UPEC infection (Figure 5*E*).

To probe the specific fraction of HMOs that could be key in providing host cell protection, we examined the effects of neutral, acidic HMOs and 3′ siallylactose (3′ SL), one of the most abundant sialylated oligosaccharides found in human milk [15, 44], using phopsho-p65 NF-κB as readout. Both neutral and acidic HMOs were extracted from pooled HMOs as described in the Methods. Whereas acidic HMOs exerted a similar effect as pooled HMOs to block p65 activation, neutral HMOs failed to block phosphorylation of the p65 transcription factor (Figure 5*F*). However, 16 hours of treatment with 1 mg/mL of 3′SL was in and of itself effective in suppressing p65 NF-kB activation (Figure 5*E*). These results support the concept that sialic acid in HMOs is involved in eliciting cellular protection during infection.

DISCUSSION

HMOs are increasingly recognized as an important innate defense strategy against many microbial infections, with studies proposing HMOs as host receptor mimics that bind to surface adhesins of microbes to interfere with their ability to interact with surface glycan receptors of epithelial cells [14–18]. Particularly, Martin-Sosa and colleagues described binding of the sialylated fraction of HMOs to UPEC, implying that HMOs hinder UPEC adherence to host cells [15]. The current paradigm is that HMOs benefit host immunity by serving as an antiadherence factor, through gentamicin protection assay; however, our study found that HMOs do not prevent UPEC adhesion, but do inhibit subsequent UPEC invasion into bladder epithelial cells. Gentamicin is a bactericidal agent that has been widely used as a standard technique to measure levels of internalized bacteria due to its poor ability to permeate eukaryotic cell membranes [29, 45]. Despite the widespread use, there are some limitations associated with gentamicin protection assay; for example, prolonged treatment and/or high concentrations of gentamicin can sometimes permeate inside eukaryotic cells to kill intracellular bacteria. To overcome this limitation, we performed all our assays using a low dosage of gentamicin (100 μg/mL) for a short period of time (2 hours), which has been routinely shown to effectively protect internalized UPEC [29]. Another potential limitation could be resistance of UPEC against this antibiotic. To ensure that none of the extracellular bacteria remained, we harvested supernatant from infected cell cultures after gentamicin treatment. With no colonies recovered from the supernatant containing gentamicin, we were able to confirm that gentamicin effectively killed extracellular UPEC and that the bacteria colonies (colony-forming units) recovered from lysed epithelial cells were from internalized population.

Interestingly, although being structurally different from HMOs, we found that GOSs had an equivalent effect to reduce UPEC invasion without affecting intracellular signaling pathways that we showed to be intercepted by HMOs. However, it remains to be investigated whether GOSs, currently added to some infant formula, are absorbed in the infant's intestine and reach the urinary tract.

Host cell detachment and apoptosis are hallmarks of UPEC pathogenesis. These events are often outcomes of focal adhesion degradation and proapoptotic signaling initiated by UPEC [25, 28, 30]. HMOs have been correlated with supporting

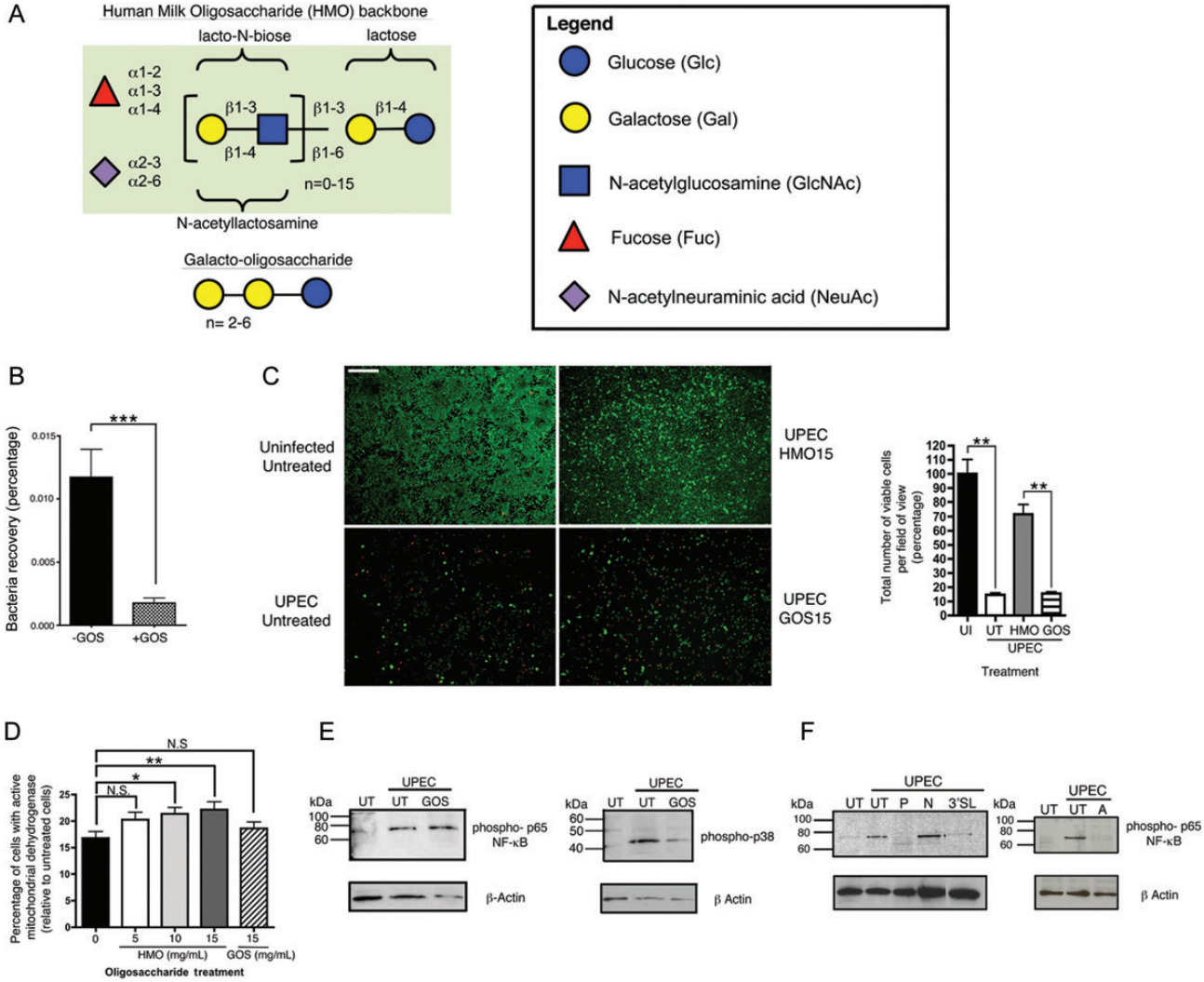

Figure 5. The sialic acid-containing fraction of human milk oligosaccharides (HMOs) is critical for inhibiting uropathogenic *Escherichia coli* (UPEC)–mediated p65 NF-κB activation. *A*, Schematic diagram of the basic molecular structures of HMOs and galactooligosaccharides (GOSs) (adapted from [13]). *B*, Invasion assay of HTB-9 cells treated with 15 mg/mL of GOS prior to UPEC infection (multiplicity of infection [MOI], approximately 25). ***$P < .001$, determined by unpaired Student *t* test. *C*, Comparison between the effect of HMOs and GOSs on cell viability during UPEC infection. Viability of live cells (green) and dead cells (red) was detected using LIVE/DEAD kit (Invitrogen) as previously described. HTB-9 cells were infected with UPEC at an MOI of 3 after 16 hours of treatment with 15 mg of HMOs (HMO15) or 15 mg of GOS (GOS15). Scale bar = 200 μm. Percentage of viability was obtained by scoring total number of viable cells (green) and divided by total number of attached cells (red and green) per field of view. *D*, Proliferation assay depicting percentage viability of HTB-9 cells pretreated with oligosaccharides prior to UPEC infection. Cells grown in 96-well plate were pretreated with 5, 10, and 15 mg/mL of HMOs or 15 mg/mL of GOSs for 16 hours prior to UPEC infection. Viability was measured using the proliferation assay WST reagent (Roche). Results represent 3 independent experimental repeats, n = 8. N.S., not significant. *$P < .02$, **$P < .01$, determined by 1-way analysis of variance analysis followed by Tukey posttest. *E*, Immunoblotting illustrating expression of phosphorylated-p65 NF-κB and phospho-p38 MAPK from HTB-9 cell lysates pretreated with GOS prior to UPEC infection. *F*, Immunoblotting of phosphorylated-p65 from HTB-9 cell lysates that were pretreated with 15 mg/mL of pooled HMOs (P), nonsialyated HMOs (N), sialylated HMOs (A), or 1 mg/mL of sialyllactose (3′SL) for 16 hours prior to UPEC infection. Actin was used as loading control. UI indicates uninfected cells; UT indicates untreated cells.

appropriate and healthy intestinal development in neonates by modulating the normal growth, differentiation, and apoptosis of intestinal epithelial cells [24], but it remained unknown whether HMOs promote cellular survival during infections. We found that HMOs markedly reduced UPEC-induced cytotoxicity. Specifically, HMOs significantly dampened cell death and cell exfoliation in a dosage- and time- dependent manner, although independent of caspase-mediated and mitochondria-mediated programmed cell death pathways.

Cell–cell adhesion and epithelial structural integrity are governed by an array of cytoskeletal components, including different types of focal adhesion molecules and cellular junction

structures [46]. UPEC causes degradation of focal adhesion molecule paxillin, resulting in rapid cell detachment. We revealed that HMOs impede cell detachment by preventing degradation and disassembly of several key focal adhesion molecules.

The roles of focal adhesion factors extend beyond maintenance of cellular adhesion structure integrity. β1 integrin plays an integral role in triggering downstream p38 and Erk1/2 MAPK activation by recruiting multiple focal adhesion proteins in nonphagocytic cells [47–50]. In addition, loss of FAK-paxillin interaction induces cell death via an Erk1/2 MAPK-dependent manner [47, 48]. Our study reveals an intricate relationship between focal adhesion, MAPK, and cell death during UPEC infections. We showed that rapid cell death and lifting during UPEC infection coincides with an extensive disruption of focal adhesion complex, along with a dramatic increase in MAPK and NF-κB activation. In parallel to this, we also observed a synergistic effect conferred by HMOs in preventing p38 and Erk1/2 MAPK activation. One plausible mechanism is that upon UPEC-mediated degradation of focal adhesion complex, MAPK pathways become activated, stimulating activation of p65 NF-κB. By preventing degradation of focal adhesion proteins, HMOs block MAPK stimulation, thereby dampening downstream p65 NF-κB signaling. In agreement with this hypothesis, we showed that GOSs, which also block UPEC invasion, do not prevent cell injury nor block p38 MAPK and p65 NF-κB activation caused by UPEC. These findings emphasize the unique property of HMOs in generating host cell protection from UPEC-induced damage, by antagonizing specific host cell signals triggered by UPEC.

In conclusion, we have described important roles played by HMOs in inhibiting UPEC invasion and cell cytotoxicity. By analyzing the status of multiple signaling pathways and cellular adhesion complexes, we demonstrated that HMOs intercept host intracellular signals exploited by UPEC to cause cell damage. These results provide significant insights into how HMOs prevent the establishment of microbial infection and support a healthy urinary tract in breast-fed infants. The inhibitory properties of HMOs could be exploited in molecular therapeutics to reduce bladder epithelial damage in UTIs.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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