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SHORT COMMUNICATION

Tamm–Horsfall glycoprotein engages human Siglec-9 to modulate neutrophil activation in the urinary tract

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Urinary tract infections are a major problem in human medicine for which better understanding of native immune defenses may reveal new pathways for therapeutic intervention. Tamm–Horsfall glycoprotein (THP), the most abundant urinary protein, interacts with bacteria including uropathogenic *Escherichia coli* (UPEC) as well host immune cells. In addition to its well-studied functions to antagonize bacterial colonization, we hypothesize that THP serves a critical host defense function through innate immune modulation. Using isolated human neutrophils, we found that THP binds neutrophils and that this interaction reduces reactive oxygen species generation, chemotaxis and killing of UPEC. We discovered that THP engages the inhibitory neutrophil receptor sialic acid-binding Ig-like lectin-9 (Siglec-9), and mouse functional ortholog Siglec-E, in a manner dependent on sialic acid on its N-glycan moieties. THP-null mice have significantly more neutrophils present in the urine compared with wild-type mice, both with and without the presence of inflammatory stimuli. These data support THP as an important negative regulator of neutrophil activation in the urinary tract, with dual functions to counteract bacterial colonization and suppress excessive inflammation within the urinary tract.
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INTRODUCTION

Urinary tract infections (UTIs) are a major medical burden in the United States, especially for women and the elderly. Uropathogenic strains of *Escherichia coli* (UPEC) are by far the most common etiological agent of UTI, causing severe bladder infection (cystitis) and acute kidney infections (pyelonephritis).[1] UTI onset frequently involves an underlying dysfunction of host defenses, including pathogen recognition and antimicrobial factors, coupled with effective pathogen-specific virulence properties of invasiveness and immune resistance.

Tamm–Horsfall glycoprotein ((THP) or uromodulin) is the most abundant protein in urine[2] and is expressed exclusively in the thick ascending loop of Henle of the kidney. By weight, this ~85 kDa protein is composed 30% of glycans, consisting of N-linked high-mannose sequences and di-, tri- and tetra-antennary complex-type N-glycans that are sialylated (including the Sda-determinant), fucosylated or sulfated.[3] O-linked glycan chains have also been reported on THP.[4]

THP has a role in antagonizing UPEC colonization of the urinary tract along with less well-studied immunomodulatory effects. THP directly binds S-fimbriated and type 1 fimbriated UPEC[5] without exerting direct bactericidal or bacteriostatic activity. High mannose glycans of THP interact with UPEC fimbrial tip protein FimH, preventing the bacterium from binding the uroepithelial receptors uroplakin Ia and Ib, thus limiting colonization of the bladder.[6] THP also inhibits fimbriated UPEC adherence to cultured renal epithelial cells,[7] and the Sda determinant present on its N-glycans prevents UPEC colonization of renal epithelium.[8]

An independent discovery of 'uromodulin' in 1985 by Muchmore and Decker,[9] subsequently recognized to represent THP, described a role in suppression of T-cell proliferation. Since then, focused studies on the interactions between purified THP and host immune cells and proteins *in vitro* have yielded a mixed array of pro-inflammatory and anti-inflammatory phenotypes. For example, dependent on the model system chosen, THP has been reported to suppress[10] or enhance[11] neutrophil phagocytosis and block nuclear factor-κB activation and cytokine release from kidney cells[12] while stimulating cytokine production from monocytes.[13] In 2004, two independently derived THP knockout ($THP^{-/-}$) mouse lines were generated and demonstrate a consistent protective effect of THP on the bladder during UTI.[14,15] Recently, it has been suggested that THP may regulate bone marrow granulopoiesis, with THP deficiency promoting systemic neutrophilia.[16]

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In this study, we examined new mechanistic aspects of THP modulation of the host immune response using neutrophils, the first innate immune responders to UTI pathogens. THP exerted a suppressive effect on neutrophil activation, including reduced chemotaxis, reactive oxygen species (ROS) production and bactericidal activity against UPEC. Blunting of neutrophil activation was secondary to THP engagement of the inhibitory neutrophil receptor sialic acid-binding Ig-like lectin-9 (Siglec-9), an interaction that depended on terminal sialic acids on the THP glycoprotein. THP thus has dual functions during UTI—its documented ability to directly bind and interfere with colonization potential of the global pathogen UPEC, coupled to Siglec-9-mediated counter-regulation of neutrophil activation to mitigate against excessive inflammation and host tissue damage.

RESULTS

THP binds neutrophils and suppresses their activation and function

$THP^{-/-}$ mice have increased urine and bladder burdens upon challenge with various pathogens, including UPEC.[14,15] We hypothesized that host inflammatory responses are altered in the absence of THP and examined the impact of THP on neutrophils, the first immune cell responders to UTI. THP binds neutrophils and alters phagocytosis at physiological concentrations found in urine and plasma,[10,11,17] but there is currently no firm mechanistic basis regarding the molecular events governing THP and neutrophil interactions. We incubated isolated human neutrophils with a physiological urinary concentration of purified THP (50 μg ml^{-1}) for 30 min prior to assessing function. Neutrophils pretreated with THP were stained with a fluorescein isothiocyanate (FITC)-labeled, mouse anti-human THP antibody and analyzed via flow cytometry, revealing a strong fluorescent signal indicative of THP binding (Figure 1a). THP pretreatment markedly reduced ROS production following stimulation with phorbol 12-myristate 13-acetate, a potent activator of protein kinase C (Figure 1b). Using a Transwell cell migration model, THP exposure strongly inhibited neutrophil chemotaxis in response to the chemoattractant N-formylmethionyl-leucyl-phenylalanine (fMLP), reducing migrated cells to the background number seen in the absence of fMLP (−control, Figure 1c). Finally, THP pretreatment inhibited neutrophil killing of UPEC (Figure 1d). THP exerted these inhibitory effects without altering neutrophil viability under our experimental conditions (30 min treatment) and by 90 min demonstrated a protective effect as determined by propidium iodide uptake (Supplementary Figure 1A). THP did not show direct antimicrobial activity against UPEC in bacterial medium (Luria-Bertani (LB) broth) or eukaryotic medium (RPMI 1640) (Supplementary Figure 1B).

THP engages the inhibitory neutrophil receptor Siglec-9

Previous work showed that THP interaction with the lymphocyte cell surface depended on N-glycan modifications[18] and that THP engaged an unknown surface glycan-binding receptor on human neutrophils in a manner outcompeted by exogenous sialic acid.[17] These clues, combined with our results revealing a consistent THP-mediated suppression of neutrophil function, led us to examine whether THP interacts with Siglecs. CD33-related Siglecs are inhibitory cell surface receptors present on multiple immune cell types that recognize sialic acids as 'self-associated molecular patterns' and limit cell activation by recruitment of inhibitory SHP family phosphatases to their intracellular domains.[19] Using a plate-based assay, we examined binding of immobilized human THP to multiple chimeric human Siglec-Fc proteins, containing the extracellular domains of the molecules. THP specifically bound human Siglec-9 but not Siglec-5, Siglec-6, Siglec-8 or Siglec-14 (Figure 1e). Siglec-9 is highly expressed on neutrophils (where it is the most abundant Siglec) and monocytes,

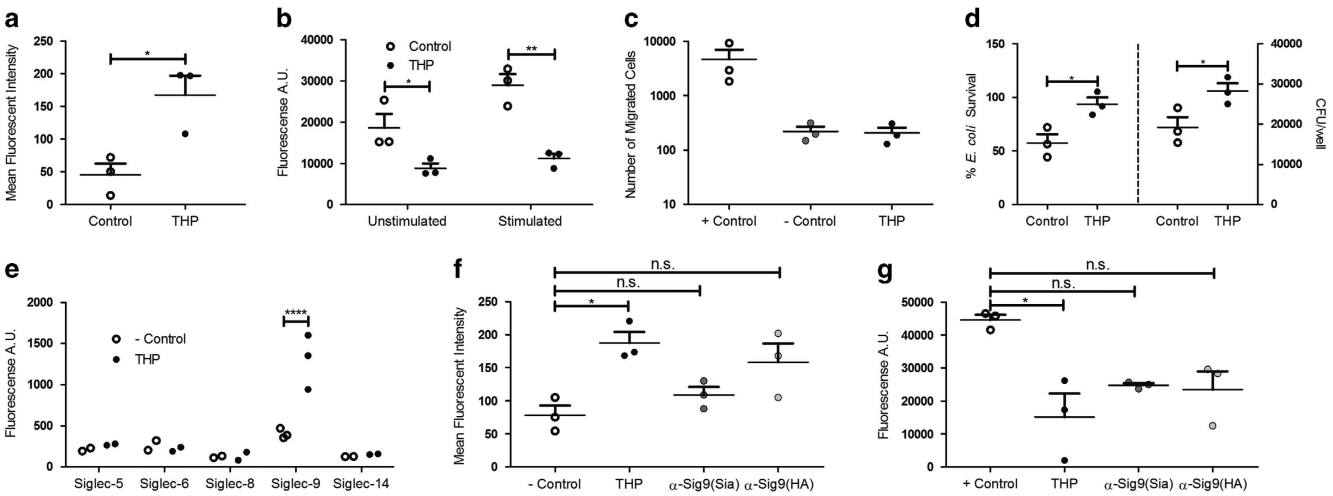

Figure 1 THP suppresses neutrophil function through engagement of Siglec-9. (**a**) Mean fluorescent intensity of neutrophils pretreated with THP (50 μg ml^{-1}) or not (control), stained with FITC mouse anti-human THP antibody. (**b**) ROS production of neutrophils pretreated with THP or not (control), with or without phorbol 12-myristate 13-acetate stimulation. (**c**) Transwell migration of neutrophils, pretreated with THP or not (+control), in response to chemoattractant fMLP. Background neutrophil migration was recorded in the absence of fMLP (−control). (**d**) Percentage of survival (left) and recovered CFU (right) of UPEC UTI89 after 30 min of exposure to neutrophils pretreated with THP or not (control). (**e**) Plate-based binding assays of immobilized THP with human Siglec-Fcs and visualized with PE anti-human IgG Fc antibody. (**f**) Mean fluorescent intensity (**f**) or ROS production (**g**) of neutrophils pretreated with mouse anti-human Siglec-9 (sialic acid blocking, Sia) or anti-human Siglec-9 (hyaluronic acid blocking, HA) antibodies and treated with THP or not (control). Data represent the mean of two independent experiments performed in technical triplicate with combined results, $n=2$ per group (**e**), or three independent experiments performed in technical triplicate with the mean and s.e.m. of combined results, $n=3$ per group (all other panels). Data were analyzed using Student's unpaired two-tailed *t*-test (**a**, **d**), two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons posttest (**b**, **e**) or one-way ANOVA with Bonferroni's multiple comparisons posttest (**c**, **f**, **g**). $^{*}P<0.05$, $^{**}P<0.01$ and $^{****}P<0.0001$ represent statistical significance, or NS represents non-significant ($P>0.05$).

with weaker expression on subsets of B cells, T cells and natural killer cells.[20] THP/Siglec-9 interactions were confirmed by flow cytometry: an anti-Siglec-9 antibody, α-Sig9(Sia), that prevents Siglec-9/sialic acid interactions blocked THP/neutrophil binding, while another anti-Siglec-9 antibody, α-Sig9(HA), that prevents Siglec-9/hyaluronic acid (HA) but not sialic acid interactions[21] blocked THP/neutrophil binding to a lesser degree (Figure 1f). These results demonstrate that Siglec-9 serves as the primary neutrophil receptor for THP and points toward THP terminal sialic acids as key constituents of this interaction. Siglec-9 engagement of sialic acid ligands can suppress neutrophil oxidative burst,[21] and indeed, we observed a partial reversal of THP-mediated suppression of ROS in neutrophils exposed to α-Sig9(Sia) or α-Sig9(HA) prior to THP treatment (Figure 1g).

THP interactions with Siglec-9 are sialic acid dependent

Siglec-9 ligands are typically glycans on host or bacterial cell surface glycoproteins and glycolipids containing sialic acid, although host and bacterial hyaluronan has recently been shown to bind human Siglec-9 as well.[21] Siglec-9 binds the glycan sequence Neu5Acα2-3Galβ1-4GlcNAc present on host cell surfaces and the exopolysaccharide capsule of the bacterium group B *Streptococcus*,[22] and this same sequence is present on THP N-glycans as a terminal structure and as a portion of the Sda determinant Neu5Acα2-3(GalNAcβ1-4)Galβ1-4GlcNAc. We analyzed the contribution of THP terminal sialic acids, including the Sda determinant, to binding of Siglec-9-Fc chimera immobilized on a microtiter plate. Binding of Siglec-9 by soluble THP was blocked ~90% by α-Sig9(Sia) prior to THP treatment but only minimally impacted with α-Sig9(HA) (<15%) (Figure 2a). When the arginine residue at position 120 of Siglec-9, critical for ligand binding of NeuAcα2-3Galβ1-4GlcNAc, was mutated to lysine (Siglec-9-R120K), THP binding was reduced to background levels (Figure 2b). Furthermore, mild periodate oxidation using $NaIO_4$ of the side chains of THP sialic acids followed by aldehyde quenching with 4-methyl-3-thiosemicarbazide (MTSC) completely abrogated THP binding to Siglec-9 (Figure 2b). Using flow cytometry, we found that $NaIO_4$-treated THP did not bind primary human neutrophils (Figure 2c). THP treatment with *Arthrobacter ureafaciens* sialidase (AUS) did not significantly alter neutrophil binding, but this may be the result of compensatory neutrophil binding to newly exposed underlying glycans (for example, galactose) via alternate receptors (Figure 2c). Finally, we observed that the inhibitory activity of THP on neutrophil ROS production and killing of UTI89 was partially or completely abolished with modification of THP terminal sialic acid through mild periodate oxidation or enzymatic removal of sialic acid with sialidase treatment (Figures 2d and e, Supplementary Table 1). Treatment of THP with β-hexosaminidase from *Canavalia ensiformis* (jack bean) to remove GalNAcβ1-4, a terminal structure on the Sda determinant, did not alter binding to Siglec-9-Fc nor was there an additive reduction of binding when combined with AUS treatment (Supplementary Figure 1C). Activity of β-hexosaminidase and AUS were confirmed under the assay conditions (data not shown).

THP regulates neutrophil populations in the urinary tract

To assess the impact of THP modulation of neutrophils *in vivo*, we first confirmed that purified mouse THP bound mouse Siglec-E, the murine functional ortholog of Siglec-9 (Figure 3a). Similar to Siglec-9, murine Siglec-E is expressed on neutrophils and peritoneal macrophages, as well as subsets of natural killer cells and dendritic cells.[23]

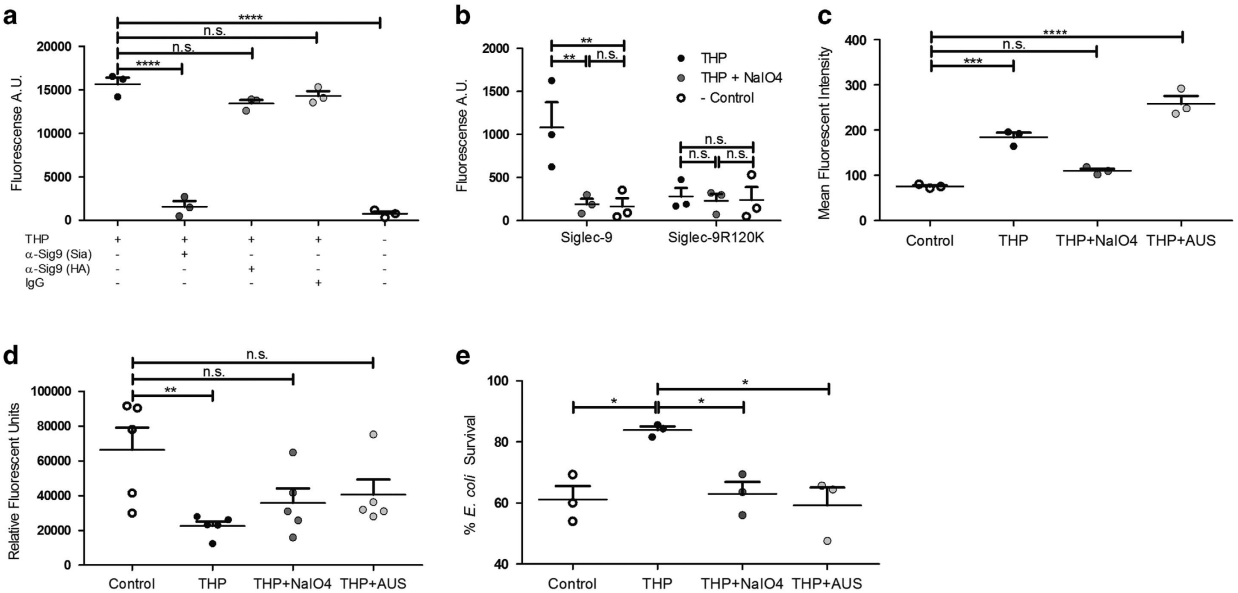

Figure 2 THP engagement of Siglec-9 and neutrophil suppression requires sialic acid. (**a**) Plate-based binding assay of immobilized human Siglec-9-Fc blocked with anti-Siglec-9 (sialic acid blocking, Sia), anti-Siglec-9 (hyaluronic acid blocking, HA) or human IgG and incubated with soluble THP. (**b**) Plate-based binding assay of immobilized mock-treated THP, $NaIO_4$-treated THP or no THP (control), with human Siglec-9-Fc or Siglec-9 R120K-Fc mutant and visualized with PE anti-human IgG Fc antibody. (**c**) Mean fluorescent intensity of neutrophils treated with THP, sialidase-treated THP or no THP (control), stained with FITC mouse anti-human THP antibody. (**d**) ROS production of neutrophils treated with THP, $NaIO_4$-treated THP, sialidase-treated THP or no THP (control) and stimulated with phorbol 12-myristate 13-acetate. (**e**) Percentage of survival (left) or total CFU (right) of *E. coli* UTI89 after 30 min of exposure to neutrophils pretreated with THP, $NaIO_4$-treated THP, sialidase-treated THP or no THP (control). Data represent the mean and s.e.m. of three (or five in (**d**)) independent experiments performed in technical triplicate with combined results shown, $n=3$ per group (**a**–**c**, **e**) or $n=5$ per group (**d**). Data were analyzed using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons posttest (**a**, **c**–**e**) or two-way ANOVA with Bonferroni's multiple comparisons posttest (**b**). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ represent statistical significance, or NS represents non-significant ($P>0.05$).

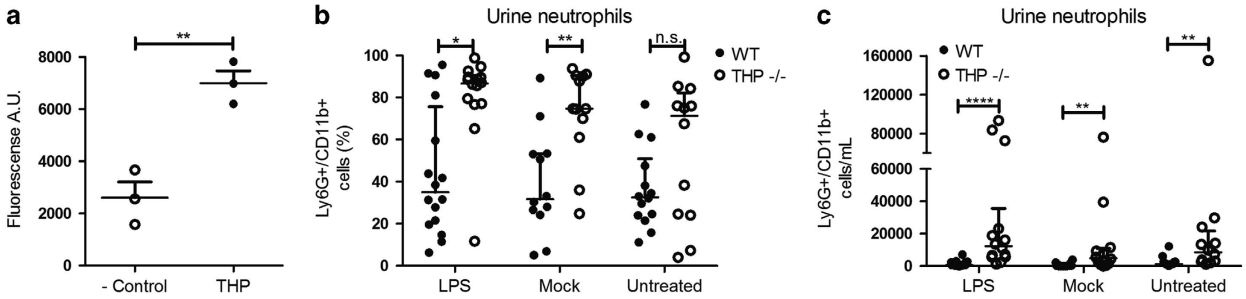

Figure 3 THP binds mouse Siglec-E and regulates urinary neutrophil populations in mice. (**a**) Plate-based binding assay of immobilized mouse THP with mouse Siglec-E-Fc and visualized with PE anti-human IgG Fc antibody. Percentage of (**b**) or total (**c**) Ly6G+/CD11b+ cells in mouse urine collected 24 h posttreatment with LPS and quantified via flow cytometry. Experiments were performed independently three times, and combined results are shown. Data represent the mean and s.e.m. of three independent experiments performed in technical triplicate, $n=3$ per group (**a**). Circles represent individual mice and lines represent the median and interquartile ranges of each group, $n=12$–16 per group (**b**, **c**). Data were analyzed using Mann–Whitney test, two-tailed (**b**, **c**), or using Student's unpaired two-tailed *t*-test (**a**). $^{*}P<0.05$, $^{**}P<0.01$ and $^{****}P<0.0001$ represent statistical significance, or NS represents non-significant ($P>0.05$).

Indirect bacterial antagonism of THP has been documented previously in prevention of bladder colonization,[6–8] although THP does not exert direct bactericidal/static activity, a finding confirmed in this study (Supplementary Figure 1B). Because of the inherent antibacterial properties of THP in wild-type (WT) mice, we examined neutrophil responses to a static bacterial stimulus, purified lipopolysaccharide (LPS), to avoid the confounding factor of increased bacterial numbers in $THP^{-/-}$ urinary tract over the experimental time course. Mice received a transurethral dose of purified LPS, and 24 h later, urine and bladders were collected. Flow cytometric analysis revealed a higher percentage of neutrophils (percentage of Ly6G+/CD11b+ of gated cells) in urine of $THP^{-/-}$ mice in all groups; LPS treated, mock treated, and untreated (not significant) (Figure 3b). Additionally, total neutrophil numbers per ml of urine were significantly increased in $THP^{-/-}$ mice in all the groups (Figure 3c).

DISCUSSION

Although several earlier studies described THP binding to human neutrophils,[10,11,17] ours is the first to identify the primary receptor as Siglec-9, in a manner where functional THP–neutrophil interactions depend upon the terminal sialylated N-glycan structures of the abundant urinary glycoprotein. The significance of THP glycan moieties for biological activity has also been observed in interaction with other immune system components. THP glycans are required for direct interactions with interleukin-1 and T-cell immunosuppression can also be attributed to its purified N-glycan portion, in the absence of intact protein.[24] However, this glycan-mediated lymphocyte immunosuppression is enhanced by stepwise enzymatic removal of sialic acids and underlying beta-galactose residues and thus localized to the central core structure of the THP N-glycan.[18] In contrast, we observed that removal or side chain truncation of the terminal sialic acid on the THP N-glycan eliminated Siglec-9 binding and reversed neutrophil suppression, consistent with a reported finding in which exogenous sialic acid outcompeted THP binding to neutrophils.[17]

Changes in THP sialylation appear to be important in various disease associations. Although total levels of THP are unchanged, patients with interstitial cystitis have only half of the THP sialic acid content of healthy controls.[25] Similarly, mass spectrometry of urine THP from kidney stone patients revealed >20% less sialic acid content than healthy controls.[26] Patients with type I diabetes are more susceptible to UTI, have more glucose and less sialic acid in their THP glycans than healthy controls[27] and may exhibit reduced THP urinary excretion rates.[28] Although reduced THP *N*-acetylgalactosamine (GalNAc), another component of the Sda antigen, has also been reported in patients with UTI,[29] terminal GalNAcβ1-4 did not contribute to THP/Siglec-9 interactions (Supplementary Figure 1C).

Allelic variants of THP correlate both with pathogen diversity and frequency of antibiotic-resistant UTI, with the *UMOD* ancestral allele, which drives higher urinary THP, retained at higher frequency because of its protective effect against UTIs.[30] Furthermore, these authors observed an inverse correlation between THP levels and leukocytes in human urine; an effect that we corroborated experimentally *in vivo* (Figure 3). Neutrophils pretreated with THP showed reduced UPEC killing, but this inhibitory phenotype was abrogated when THP sialic acid was modified or removed (Figure 2). Thus THP-mediated neutrophil suppression comes with a cost—a diminished neutrophil contribution to bacterial clearance. However, as very few immune cells are typically found in healthy urine, and THP directly binds UPEC to impede bladder colonization,[6–8] we hypothesize that a dual role to limit inflammatory responses and potential damage to the vulnerable kidney is advantageous.

Collectively, our data demonstrate that THP can bind neutrophil Siglec-9 to control excessive neutrophil inflammatory responses. This interaction requires the sialic acid present on THP, and varying glycosylation patterns of THP may explain susceptibility of certain individuals to recurrent UTI. Future work seeks to examine THP-Siglec signaling during UTI as a potential therapeutic target to prevent or treat acute and chronic UPEC UTI.

METHODS

Bacterial strains and growth conditions

WT UPEC strain UTI89 (O18:K1:H7)[31] was incubated overnight to stationary phase at 37 °C with shaking in LB broth, and overnight cultures were diluted 1:30 in fresh LB broth and incubated shaking at 37 °C until mid-log phase ($OD_{600nm}=0.4$).

Primary neutrophil isolation

With approval from UCSD IRB/HRPP, protocol no. 070278X, human venous blood was obtained from 12 healthy volunteers under informed consent, with heparin used as an anticoagulant. Neutrophils were isolated using Polymorph-Prep (Axis-Shield, Dundee, Scotland) to create a density gradient by centrifugation according to the manufacturer's instructions.

THP purification and modification

Purified human pooled THP was purchased from BBI Solutions, Cardiff, UK (Catalog number: P135-1). Mouse THP was purified from pooled mouse urine as described originally by Tamm and Horsfall[2] using salt precipitation with several modifications. Pooled mouse urine was diluted with an equal volume of

chilled distilled H_2O, and subsequent precipitation and desalting steps were carried out at 4 °C. THP was precipitated by adding $NaCl_2$ to a final concentration of 0.58 M, and mixed and the precipitate was allowed to settle overnight. The precipitate was pelleted at 3220 *g* for 10 min, supernatant was discarded and the pellet was washed with fresh, chilled 0.58 M $NaCl_2$. Sample was vortexed, and the precipitate was again allowed to settle overnight and pelleted as in the previous step. Pellet was resuspended in three volumes of distilled H_2O and desalted using a 50 kDa Amicon Ultra-15 column (EMD Millipore, Billerica, MA, USA) and a minimum of 3× buffer exchanges with distilled H_2O. After desalting and concentrating to 1 ml, the sample was centrifuged at 9300 *g* for 30 min, and any pelleted impurities were discarded. Mouse THP was quantified via BCA assay (Pierce, Rockford, IL, USA). A single band (molecular weight ~85 kDa) was visualized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis after staining with InstantBlue (Expedeon Inc., San Diego, CA, USA) and positively identified via western blotting following incubation with a goat anti-THP polyclonal antibody (Cat. no. sc-19554, Santa Cruz Biotechnology, Dallas, TX, USA).

To remove or modify the sialic acid on THP for binding assays, human THP was exposed to either sialidase treatment or mild periodate oxidation. For sialidase treatment, THP (100 μg ml^{-1}) was incubated with 100 mU ml^{-1} of sialidase purified from AUS (Sigma Aldrich, St Louis, MO, USA) in 1× Dulbecco's phosphate-buffered saline (DPBS; pH = 7.0) for 1 h at 37 °C. Sialidase activity was confirmed using a Neuraminidase Assay Kit (Abcam, ab138888, Cambridge, UK). Selective periodate oxidation of THP sialic acid-containing glycans was accomplished by incubating purified THP (200 μg ml^{-1}) with fresh 2 mM $NaIO_4$ (Sigma Aldrich) on ice for 20 min to generate aldehydes at the C7 or C8 position of sialic acid. To stop the reaction, $NaIO_4$ was removed by transferring sample to a 30 kDa Microcon column (EMD Millipore), centrifuged for 15 min at 14 000 *g* at 4 °C and washed 3× with ice-cold 1× DPBS. Sialic acid aldehydes were then quenched with MTSC (Sigma Aldrich) as performed previously.[32] Subsequent treatment with FITC-thiosemicarbazide verified that all aldehydes were fully quenched under assay conditions (data not shown).

Neutrophil flow cytometry

Freshly isolated human neutrophils (6×10^5 cells ml^{-1}) were incubated with THP (50 μg ml^{-1}) in Hank's Balanced Salt Solution (HBSS) with calcium and magnesium for 15 min on ice. Where indicated, neutrophils were incubated with 1 μg ml^{-1} mouse anti-human Siglec-9 monoclonal antibody (α-Sig9(Sia), Cat. no. MAB1139, R&D Systems, Minneapolis, MN, USA) or mouse anti-human Siglec-9 monoclonal antibody (α-Sig9(HA), Cat. no. 624084, BD Pharmingen, San Diego, CA, USA) for 15 min on ice prior to incubation with THP. Non-bound THP was removed by centrifuging at 200 *g* for 5 min and washing once with HBSS. Cells were then incubated with 1 μg ml^{-1} FITC mouse anti-human THP antibody (Cat. no. AM31843FC-N, Acris, Rockville, MD, USA) for 15 min on ice. Cells incubated with the anti-human THP antibody only were used as a negative control. Cells were washed once with HBSS and run on BD FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analyzed using the CellQuest Pro v.6 software (BD Biosciences).

Neutrophil killing assay

Neutrophils were diluted to 2×10^6 cells ml^{-1} in RPMI 1640 (Gibco, Cat. no. 11875-093, Grand Island, NY, USA), treated with THP (50 μg ml^{-1}) and incubated at 37 °C in 5% CO_2 for 30 min. Untreated neutrophils were used as a control. Neutrophils were seeded at 2×10^5 cells per well in a tissue cultured-treated 96-well plate. UTI89 diluted in RPMI 1640 was added to neutrophils at a multiplicity of infection of 1:1 (UTI89-to-neutrophil ratio). Control wells without neutrophils were used to determine baseline bacterial counts at the assay end point. Plates were centrifuged at 300 × *g* for 5 min to facilitate bacterial contact with neutrophils and incubated at 37 °C in 5% CO_2 for 30 min. Samples were lysed, serially diluted and then plated on LB agar for enumeration of surviving UTI89 colony-forming unit (CFU). Percentage of survival of UTI89 was calculated as ((CFU per experimental well)/(CFU per control well)) × 100.

ROS production assay

Neutrophils were stained in HBSS without calcium and magnesium containing 20 μM 2′,7′-dichlorofluorescein diacetate (Sigma Aldrich) and were incubated with THP (50 μg ml^{-1}) for 30 min at 37 °C with 5% CO_2. Where indicated, neutrophils were incubated with 1 μg ml^{-1} mouse anti-human Siglec-9 monoclonal antibody α-Sig9(Sia) or mouse anti-human Siglec-9 monoclonal antibody α-Sig9(HA) for 15 min prior to incubation with THP. Cells were then added to a 96-well plate (5×10^5 cells per well) and mixed at a 1:1 ratio with 25 nM phorbol 12-myristate 13-acetate (Sigma Aldrich) to stimulate ROS release. Plates were incubated at 37 °C with 5% CO_2 for 30 min in the dark. Fluorescence intensity (485 nm excitation, 530 nm emission) was measured in a SpectraMAX Gemini EM fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

Transwell chemotaxis assay

Neutrophils (2×10^6 cells ml^{-1}) were incubated with THP (50 μg ml^{-1}) for 30 min at 37 °C with 5% CO_2. Cells were seeded in six-mm transwell permeable supports (3-μm pore size; Corning Inc., Corning, NY, USA) placed in 24-well plates. Lower chambers contained either HBSS alone or 100 nM of the chemoattractant fMLP (Sigma Aldrich). Following a 45-min incubation at 37 °C with 5% CO_2, inserts were removed, the cells were suspended with gentle pipetting and 5 mM EDTA was added. Samples were immediately run on BD FACSCalibur (BD Biosciences) and gated based on forward and side scatter profiles of input (isolated neutrophils), and data were analyzed using the FlowJo v10.2 software (FlowJo LLC, Ashland, OR, USA).

Siglec-Fc binding assay

Chimeric recombinant Siglec-Fc fusion proteins of human Siglec-5, Siglec-6, Siglec-8, Siglec-9 (WT and R120K) and Siglec-14 or mouse Siglec-E extracellular domains and a human IgG Fc tail were generated as described previously.[33] For all binding assays except those using anti-Siglec-9 antibodies, THP (10 μg per well) was immobilized on high affinity binding microtiter plates (Corning, Catalog number 3361) in 1× DPBS overnight at 4 °C. Uncoated wells were used as negative controls and subjected to all subsequent steps. Wells were blocked for 1 h with 1% bovine serum albumin (BSA) and incubated with the indicated Siglec-Fc constructs (1 μg per well) for 1 h, and the wells were then incubated with 0.5 μg per well PE anti-human-IgG Fc antibody (Clone HP6017, Cat. no. 409304, BioLegend, San Diego, CA, USA). Fluorescence intensity (546 nm excitation, 578 nm emission) was measured on a SpectraMAX Gemini EM fluorescence plate reader.

For binding assays using anti-Siglec-9 antibodies, Protein A-coated microtiter plates (Cat. no. 15155, Pierce) were incubated with Siglec-9-Fc (1 μg per well) in 1× DPBS overnight at 4 °C. Wells were blocked for 1 h with 1% BSA and incubated for 1 h with 10 μg ml^{-1} mouse anti-human Siglec-9 monoclonal antibody α-Sig9(Sia), mouse anti-human Siglec-9 monoclonal antibody α-Sig9 (HA), recombinant human IgG (non-specific control, Bio-Rad) or 1% BSA only (for positive and negative controls). Wells were subsequently incubated with THP (10 μg per well) in 1% BSA with PBS at room temperature for 1 h, followed by 0.5 μg per well FITC mouse anti-human THP antibody in 1% BSA with PBS for 30 min. Fluorescence intensity (494 nm excitation, 520 nm emission) was measured on a SpectraMAX Gemini EM fluorescence plate reader.

Mouse LPS cystitis model

All studies involving animals were reviewed and approved by the University of California San Diego Animal Care and Use Committee and performed using accepted veterinary standards. $THP^{+/+}$ (WT) and $THP^{-/-}$ mouse breeding pairs, described previously,[15] were a generous gift from the Kumar laboratory and bred and maintained at UCSD. All animals used in this study were females aged 2–4 months. Mice were allowed to eat and drink *ad libitum*. All efforts were made to minimize suffering of animals employed in this study.

To induce cystitis with purified *E. coli*, LPS (Sigma Aldrich) was suspended at 1 mg ml^{-1} in molecular grade water. LPS was diluted to 100 μg ml^{-1} in tissue culture-grade sterile DPBS, and 50 μl was introduced into the bladder through transurethral insertion of a 30 G 1/2 inch hypodermic needle catheter encased in an UV-sterilized polyethylene tube (inner dimension 0.28 mm, outer dimension 0.61 mm, Catalog no.598321, Harvard Apparatus, Holliston, MA, USA) into an isoflurane-anesthetized mouse. The bladder was voided of urine prior to LPS introduction. Mock-treated animals received 50 μl of sterile DPBS via catheter. Urine was collected 24 h posttreatment.

Flow cytometry of urine

Urine samples were passed through a 40-μm filter, and cells were washed in PBS and blocked with 2% fetal bovine serum for 15 min on ice. Staining of surface markers was performed in 2% fetal bovine serum using 0.5 μg ml^{-1} anti-CD11b-FITC (Clone M1/70, Cat. no. 553310, BD Pharmingen) and anti-Ly6G-APC (Clone 1A8, Cat. no. 127614, BioLegend) for 30 min on ice. Samples were gated on unstained cells and positive signals were determined using single-stain controls. Samples were run on BD FACSCantoII (BD Biosciences) and data were analyzed using the FlowJo v10.2 software (FlowJo LLC).

Statistical analyses

All *in vitro* experiments were performed in technical triplicates and repeated in at least three independent experiments. Statistical analyses were performed on the means of independent experiments. All neutrophil assays were performed with at least three biological (donor) replicates. All *in vivo* experiments were performed using at least four mice per group and repeated in three independent experiments with results combined prior to statistical analyses. For *in vitro* experiments, sample size to ensure adequate power to detect effects was based on prior similar studies performed by our group and others. For *in vivo* experiments, sample size was estimated using the Power/Sample Size Calculator provided by the University of British Columbia Department of Statistics (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). Animals were not randomized or blinded prior to experiments. Statistical analyses were conducted using GraphPad Prism, version 5.04 (GraphPad Software Inc., La Jolla, CA, USA). For *in vitro* experiments, all data were assumed parametric and statistical analyses performed include Student's unpaired two-tailed *t*-test, one-way analysis of variance with Bonferroni's multiple comparisons posttest or two-way analysis of variance with Bonferroni's multiple comparisons posttest as indicated in the figure legends. For *in vivo* experiments, statistical analyses performed include non-parametric two-tailed Mann–Whitney test as indicated in the figure legends. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ represent statistical significance or NS represents non-significant ($P>0.05$).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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