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Targeting Deficiencies in the TLR5 Mediated Vaginal Response to Treat Female Recurrent Urinary Tract Infection.

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Summary: The identification of the host defence peptides as target effectors in the innate defence of the uro-genital tract creates new translational possibilities for immunomodulatory therapies, specifically vaginal therapies to treat women suffering from rUTI, particularly those carrying the TLR5_C1174T SNP.

39 Urinary tract infections (UTIs) are a microbial disease reported worldwide. Women are particularly
40 susceptible with many suffering debilitating recurrent (r) infections. Treatment is by antibiotics, but
41 such therapy is linked to antibiotic resistance and re-infection. This study explored the innate
42 protective mechanisms of the urogenital tract with the aim of boosting such defences therapeutically.

43 Modelling UTIs *in vitro*, human vaginal and bladder epithelial cells were challenged with
44 uropathogenic *Escherichia coli* (CFT073) and microbial PAMPs including flagellin, LPS and
45 peptidoglycan. Flagellin functioning via the TLR5/NFκB pathway was identified as the key UPEC
46 virulence factor causing a significant increase ($P < 0.05$) in the production of the host-defence peptide
47 (HDP), BD2. BD2-depleted urine samples from bladder infected mice supported increased UPEC
48 growth, strengthening the significance of the HDPs in protecting the urogenital tissues from infection.

49 Clinically, vaginal-douche BD2 concentrations were reduced ($p < 0.05$) in women suffering rUTIs,
50 compared to age-matched healthy controls with concentrations further decreased ($p < 0.05$) in a
51 TLR5^{392Stop} SNP rUTI subgroup. Topical vaginal estrogen treatment increased ($p < 0.001$) BD2
52 concentrations in all women, including those carrying the SNP. These data identify therapeutic and
53 antibiotic sparing roles for vaginal immunomodulatory agents that specifically target HDP induction,
54 facilitate bacterial killing and disrupt the UPEC infection cycle.

55

56 **Introduction**

57 Urinary tract infections (UTI), linked to *Escherichia coli* infecting the bladder are one of the most
58 prevalent microbial diseases, accounting for over eight million health-care visits yearly in the United
59 States ¹. Females are particularly susceptible ² and of those women affected 5%, rising to 13% in
60 those aged over 60, ^{3,4} will suffer from debilitating recurrent infections (rUTI). These infections not only
61 impact on a person's quality of life, but are also associated with escalating healthcare costs ¹. In a
62 number of cases the recurrent infections can be linked to an abnormality of the urinary tract ⁵, but a
63 major characteristic of the disease is the poorly-defined relationship between the host
64 genotype/phenotype and the main pathogen, uropathogenic *E. coli* (UPEC).

65 In healthy individuals, the lower urinary tract is maintained free of pathogens through the functioning
66 of host defences that include the pH and ionic composition of urine, which in conjunction with the
67 flushing action of urine are detrimental to bacterial adherence and growth. Evidence also suggests

68 that these physical factors are supported by host innate, rather than adaptive, immune defence
69 mechanisms with the high rates of UTI linked to the bladder's inability to mount an antibody
70 response⁶. Innate elements protecting the lower urinary tract include the activities of either
71 constitutively or induced host defence molecules. Antimicrobial agents such as uromodulin facilitate
72 bacterial removal by binding UPEC directly, while others, including lactoferrin and neutrophil
73 gelatinase-associated lipocalin-2, function by sequestering iron to restrict bacterial survival and
74 growth⁷. Small cationic peptides, synthesised by epithelia and neutrophils, and including the
75 defensins, cathelicidin, and ribonucleases, are also part of the urinary tract innate defences⁸. These
76 molecules, in addition to their intrinsic bacterial killing properties, fight infection through their
77 immunomodulatory properties that promote increased cytokine production and neutrophil infiltration⁹.
78 However, the actual roles of such peptides in protecting the urogenital tract from infection remain
79 contentious. Studies involving mice deficient in the antimicrobial peptides Defb1 (analogous to human
80 Beta-Defensin-1) and cathelin-related antimicrobial peptide (analogous to the cathelicidin hCAP-18)
81 report conflicting results, with the absence of peptides associated with either increased, reduced or no
82 differences in UTI susceptibility¹⁰⁻¹³.

83 Epithelial cells recognise potential pathogens through receptors including the Toll-like receptors
84 (TLRs). Once activated, the TLRs induce a rapid response that results in microbial killing either
85 directly through the synthesis and activities of antimicrobial agents or indirectly through chemokines,
86 and the attraction of neutrophils and macrophages. Studies focussed on UPEC infection of the
87 murine urinary tract suggest that TLR4 activation, linked to the detection of microbial
88 lipopolysaccharide and/or FimH, the adhesin portion of the Type 1 fimbriae that secures UPEC
89 attachment to urothelial cells, can trigger host innate antimicrobial defences¹⁴. Following urothelial
90 cell invasion, TLR4 is further implicated in the expulsion of UPEC and reinfection of the urinary tract,
91 via an exocytic pathway linked to cellular cAMP¹⁵. However, *in vitro* data using proliferating and
92 differentiated normal urothelial cells favours TLR5 signalling mechanisms predominating in the human
93 bladder¹⁶. In support, murine studies have also demonstrated bacterial motility and TLR5 to be a key
94 factors in UPEC pathogenicity, with wild-type flagellated UPEC shown to out-compete non-flagellated
95 *fliC* mutants and maximum expression of flagellar genes coinciding with UPEC ascent into the ureters
96 and kidneys^{17,18}. Clinically, patient susceptibility to UTI links to polymorphisms of both receptor genes
97 with a TLR4_A896G polymorphism related to rUTI protection, but a TLR5_C1174T SNP linked to an
98 increased susceptibility to repeated infections^{19,20}.

99 Current management strategies for rUTI sufferers are generally prophylactic involving long-term low
100 dose antibiotics. In reality such strategies provide limited long-term benefit and encourage bacterial
101 resistance that further complicates patient management. The treatment also conflicts with the current
102 public health challenges of managing and reducing antibiotic overuse^{21,22}. Increasing concerns
103 relating to the overuse of antibiotics has stimulated interest in alternative therapies including vaccine
104 development and identification of agents able to boost endogenous innate defences of the urogenital
105 tract⁸. In females the initiating event of an UTI involves colonisation of the vaginal mucosa by UPEC
106 originating, it is presumed, from the gut microbiota²³, with UTI caused by the subsequent urethral
107 migration and attachment of these bacteria to the urothelium²⁴.

108 We show here that not only does deficiency in the vaginal host antimicrobial defences link to recurrent
109 UTIs, but also that vaginal innate defences can be enhanced through topical immune boosting
110 agents. We propose that such agents can be utilised to disrupt the UPEC infection cycle and hence
111 offer an effective antibiotic-sparing approach to the treatment of rUTIs.

112 **Results**

113 ***E.coli* and Flagellin induce a TLR5-Mediated BD2 Response in Urogenital Epithelia**

114 Immortalised vaginal (VK2 E6/E7) and bladder cancer (RT4) cells modelling the urogenital epithelial
115 tissues were challenged with a heat-inactivated suspension of the motile flagellated UPEC strain
116 CFT073 and *E.coli* flagellin. Expression of genes *DEFB1*, *DEFB4*, *DEFB103A*, *LCN2*, *SLPI*, *hCAP-18*
117 and *DEF5A* encoding the host defence molecules BD1, BD2, BD3, Lipocalin2, secretory leukocyte
118 protease inhibitor (SLPI), cathelicidin and HD5 were assessed using end-point PCR. While these data
119 suggested *DEFB1* and *DEFB4* gene expression to be upregulated (**Fig 1a and Supplementary S1a-**
120 **d**), qRT-PCR analyses identified only a significant increase in *DEFB4* gene expression (**Figs 1b & Fig**
121 **S2**) with these observations accompanied by a significant increase in BD2 peptide following CFT073
122 (P<0.05) and flagellin (P<0.001) challenge (**Fig 1c**). These data were substantiated using primary
123 human ureteric urothelial and vaginal epithelial cells (**Fig 1d & e**). Hence we selected BD2, an
124 inducible bacterial killing molecule and immunomodulatory agent with reported potency in inhibiting
125 UTI progression²⁵ as our key target in exploring and devising strategies to boost the UT innate
126 defences.

127 Challenging VK2 E6/E7 and RT4 cells with flagellated (motile) and non-flagellated (non-motile)
128 *Escherichia coli* indicated that the signalling mechanism controlling BD2 production in the urogenital

129 tissues was TLR5 mediated and linked to flagellin (**Fig 1f**). This was further indicated by a reduction in
130 *DEFB4* transcript expression in flagellin challenged vaginal VK2 E6/E7 cells following siRNA silencing
131 of TLR5 gene expression (**Fig 1g**). In support decreased BD2 concentrations were also detected in
132 the media bathing the vaginal cells similarly challenged with flagellin, but in which TLR5 had been
133 blocked using antibody (**Fig 1h**). The use of RT4 bladder cells engineered to contain a NFκB-
134 luciferase reporter also indicated the NFκB signalling pathway to be involved with a four hour flagellin
135 challenge linked to a significant 44.8 fold increase in luciferase activity ($P < 0.001$) (**Fig 1i**). In
136 comparison only slight induction of NFκB activity was observed at four hours following either
137 peptidoglycan (3.1 fold) or lipopolysaccharide (LPS) challenges (6.7 fold) with maximal responses at
138 16 hours of 6.2 and 10.7 fold respectively (**Fig 1i**). However, LPS challenges of up to 24 hours *in vitro*
139 did not induce *DEFB4* expression (**FigS3**). These data identified TLR5/NFκB/BD2 as a key signalling
140 pathway in the innate response of the urogenital epithelia to a flagellated UPEC infection.

141 **Deficiency of BD2 Orthologue (DefB4) in Mice Increases Susceptibility to UTI**

142 To verify this pathway and the significance of host defence peptides including BD2 *in vivo*, C57BL/6J
143 WT and *Tlr5*^{-/-} knockout mice bladders were inoculated with either buffer (PBS), *E.coli* derived flagellin
144 (5μg) or CFT073 (10⁸) and the murine bladder and vaginal tissues examined for *DefB4* gene
145 expression. The *DefB4* gene is the mouse orthologue of the human *DEFB4* gene²⁶. C57BL/6J WT
146 mice showed a significant increase in bladder *DefB4* transcript expression ($P < 0.05$) in response to the
147 flagellin challenge (**Fig 2a**), while significant changes in both bladder and vaginal expression ($P < 0.05$)
148 were associated with the bacterial (CFT073) infection (**Fig 2a, b**). No renal *DefB4* transcript
149 expression was identified (data not shown) and no bacteria were recovered from the kidneys (**Fig 2c**).

150 The bladder and vaginal tissues of similarly challenged *Tlr5*^{-/-} mice showed no significant induction of
151 *DefB4* transcript expression (**Fig 2a, b**). At six hours post challenge the bladder bacterial counts of
152 these mice were also significantly higher than those recorded in the C57BL/6J WT mice (**Fig 2c**).
153 Additionally bacteria were recovered from the kidneys of the *Tlr5*^{-/-} mice indicating the increased
154 susceptibility of their urinary tracts to an ascending UTI (**Fig 2c**). These *in vivo* data supported the
155 significance of the urogenital epithelial TLR5/BD2 host defence peptide (HDP) signalling response in
156 the immediate protection of the lower and upper urinary tract from UPEC infection.

157

158

159 **Biological Relevance of BD2 in the Innate Response of the Uro-genital Tract**

160 To address the biological relevance of host defence peptides in the innate defence of the urogenital
161 tissues the antimicrobial potencies of an array of agents including BD2, LCN and SLPI (7.5 to 250
162 nM) were compared using a time-kill assay approach and CFT073 as the target bacterium. Using
163 peptide concentrations mimicking those at the epithelial surface^{27,28} these assay data suggested the
164 induced BD2 peptide to possess significant antimicrobial potency against the CFT073 strain (**Fig 3a**).
165 Immuno-depletion of BD2 from the wild-type mouse urine samples collected following challenge with
166 CFT073 and flagellin was also associated with increased CFT073 growth (**Fig 3b**). These data
167 provided direct evidence that a urogenital deficiency of host defence peptide links to the increased
168 risk of UTI.

169 **Women with rUTI and TLR5_C1174T SNP show Reduced BD2**

170 To examine the clinical significance of host defence peptides in defending against rUTIs we focused
171 on *DEF4B* expression and BD2 synthesis in vaginal biopsies, vaginal secretions (douche) and urine
172 samples from 86 women. Forty-eight women had a history of rUTIs, including 14 with a symptomatic
173 *E.coli* infection at time of sampling and 38 were age-matched controls. Analyses of the vaginal
174 biopsies indicated that those women suffering rUTIs showed significantly lower *DEF4B* transcript
175 expression ($p < 0.05$) compared to their respective controls (**Fig 4a**). The vaginal douche
176 measurements also supported these data with the mean BD2 concentration being significantly
177 reduced ($P < 0.05$) in the rUTI cohort (25.3 ± 3.2 pg/ml) compared to the control group (38.9 ± 5.5 pg/ml)
178 (**Fig 4b**). Stratification of these vaginal douche data also showed that the mean BD2 concentrations of
179 the pre and postmenopausal rUTI cohorts were significantly decreased ($p < 0.05$) compared to their
180 respective controls (**Fig 4c**). Furthermore, the mean BD2 concentration of the postmenopausal rUTI
181 group was significantly decreased ($p < 0.001$) compared to that of the premenopausal rUTI cohort.

182 TLR5 gene expression data suggested no significant differences between vaginal expression in the
183 control and rUTI cohorts, and expression was elevated significantly ($P < 0.05$) during active infections
184 (**Fig 4d**). These data suggested that the observed reductions in BD2 were related to cellular signalling
185 events linked to and/or downstream of TLR5. To explore this further we sequenced the TLR5 genes
186 of the patient and control cohorts. Sequencing identified a sub-group of six women (7%), all contained
187 within the rUTI group, who carried the TLR5_C1174T single nucleotide polymorphism (SNP). Also
188 known as TLR5^{392Stop}, this polymorphism is a heterozygous variant present in 5-10% of the population

189 encoding a stop-codon, which is reported to increase the susceptibility of those affected to flagellated
190 infections^{29,30}. Vaginal douche measurements also supported HDP concentrations being significantly
191 lower ($P<0.01$) in this group compared to the no SNP rUTI cohort (**Fig 4b**).

192 Of the fourteen women with an active UTI during our sample collections, nine carried the wild-type
193 TLR5 gene and five the TLR5^{392Stop} SNP. Analyses of urines and vaginal washings of these women
194 during their active infections showed that the SNP cohort had significantly lower mean BD2
195 concentrations in both urine ($P<0.05$) and vaginal douche ($P<0.001$) samples compared to those of
196 the no SNP group (**Fig 4e**). Furthermore, analysing the urine BD2 concentrations of the TLR5^{392Stop}
197 SNP cohort during periods of infection and quiescence showed no significant differences (**Fig 4f**),
198 confirming the inability of the urogenital tissues of these SNP patients to respond to infection. In
199 contrast the urine BD2 concentrations of the no SNP rUTI patients were elevated ($P<0.01$) in
200 response to infection (**Fig 4f**). However, further analyses showed that the BD2 concentrations of the
201 no SNP rUTI patients measured during an active UTI were actually very similar to those of the control
202 pre-menopausal group (**Fig 4g**). These data suggested that the vaginal BD2 responses in the no SNP
203 rUTI patients were considerably compromised, which increased their susceptibility to recurrent
204 infections. The diminished host BD2 response of the no SNP rUTI group implicated host signalling
205 defects linked to and/or downstream of TLR5.

206 To demonstrate that the rUTIs suffered by the SNP patients were due to host-susceptibility rather
207 than bacterial virulence factors, *E.coli* were isolated from the urine samples of three of the SNP
208 patients and used to challenge the stably transfected RT4-NF κ B luciferase reporter cells. Significant
209 increases in NF κ B-reporter activity comparable to flagellin (250ng/ml) were recorded following
210 challenges with each of the UPEC strains (**Fig 4h**). These data verified that the genetic TLR5^{392Stop}
211 polymorphism played a key role in predisposing these women to repeated UTIs. Modelling the
212 polymorphism experimentally by TLR5 antibody blocking of the RT4-NF κ B luciferase reporter cells,
213 prior to bacterial challenges, reduced NF κ B reporter activity (**Fig 4i**) and diminished the epithelial
214 effector IL-8 and BD2 responses (**Fig 4j, k**). Consistent with the bladder response (**Fig 4k**) and
215 patient data a reduction in BD2 was observed in similarly challenged vaginal cells (**Fig 4S**). These
216 data provided further evidence that in the urogenital tract the epithelial host **TLR5-NF κ B-HDP**
217 **signalling pathway functions** in helping to protect from UTIs and if compromised, the loss of such
218 **defences, can expose the tissues to infection.**

219 **Deficient BD2 Responses can be Enhanced Therapeutically *In Vitro* and Clinically**

220 For all rUTI patients, including those carrying the TLR5 SNP, topical agents that boost the urogenital
221 defences, including BD2 synthesis, may provide additional and/or alternative therapies to antibiotics.
222 Vaginal estrogen supplements can be used to reduce UTI risk in post-menopausal women suffering
223 rUTIs, but the mechanisms of action are not fully understood³¹⁻³³. When VK2 E6/E7 vaginal cells
224 were supplemented with 4nM estrogen for 7 days and then challenged with flagellin (50ng/ml) *DEF4B*
225 transcript expression and BD2 synthesis were significantly ($P<0.01$) potentiated (**Fig 5a, b**). These *in*
226 *vitro* data suggest that the steroid hormone, in addition to its roles in reproduction, functions in
227 augmenting the innate immune defences of the urogenital tract. When examined clinically, the
228 vaginal BD2 concentrations of post-menopausal women suffering rUTIs, but prescribed topical vaginal
229 estrogen (Vagifem 10mcg twice weekly), for a minimum of six weeks were significantly increased
230 ($P<0.001$), compared to those treated using other therapies including antibiotic prophylaxis and/or
231 advice (**Fig 5c, d**). These vaginal douche data were supported by vaginal biopsies taken from
232 estrogen-treated women that showed immunoreactivity for BD2 (**Fig 5e**). Together these data provide
233 compelling evidence that estrogen can enhance the host vaginal epithelial defences through the
234 increased synthesis of host defence molecules and help protect from rUTIs. **The fact that the BD2**
235 **vaginal douche concentrations of the rUTI TLR5 SNP patients were augmented in response to topical**
236 **vaginal estrogen treatment (Fig 5f), lends support to the use of immune-boosting vaginal therapies to**
237 **help treat rUTIs.**

238 We propose therefore that the TLR5-NF κ B-host defence effector pathway is a significant innate
239 defence mechanism helping to protect the urogenital tract against rUTI. We propose that for women
240 carrying the TLR5 SNP and hence genetically predisposed to rUTI, as well as post-menopausal
241 women, this pathway be exploited clinically. We argue that the use of topical immune modulating
242 therapies that activate and/or enhance the vaginal innate defences will help prevent bacterial
243 colonisation and ascension into the urinary tract (**Fig 5g**).

244 **Discussion**

245 UTI is one of the most prevalent microbial diseases worldwide. It is characterised by high recurrence
246 rates amongst sufferers and by the marked, and increasing multi-drug resistance profiles of
247 uropathogenic bacteria, a consequence of the mainstay therapy being limited to repeated or
248 prophylactic antibiotic treatments^{34,35}. Key challenges to science and medicine are to reduce

249 recurrence rates and improve patient outcomes through new therapeutic options that curtail antibiotic
250 usage.

251 To address this our studies focussed on both bacterial virulence factors and the host urogenital
252 defences. In support of previous reports^{16,18} our *in vitro* and murine data indicated that flagellated
253 *E.coli* through TLR5 activation play a key role in the pathology of UTI. These data were further
254 strengthened by our clinical findings, which showed that all patients carrying the TLR5_C1174T SNP,
255 encoding a stop mutation linked to increased susceptibility to flagellated bacteria²⁰, suffered from
256 recurrent UTIs. The fact that the *in vitro*, murine and clinical study data were all characterised by the
257 reduced production of host defence molecules, including bacterial killing peptides and chemokines,
258 provided a mechanistic explanation to link a TLR5 deficiency to repeated infections of the bladder.
259 Furthermore this immunodeficiency impacted similarly on the TLR5 signalling pathway of the vaginal
260 tissues. In view of this we propose that the resultant reduction in innate effector molecules facilitates
261 the repeated colonisation of the vaginal mucosa by gut *E. coli*, which drives the persistent bladder
262 infections. This mechanism also explains why antibiotic therapy can never cure women carrying the
263 TLR5^{392Stop} SNP and in fact why repeated treatments only exacerbate the condition through selecting
264 for antibiotic resistance. Hence as a first step we propose that women suffering uncomplicated rUTI
265 should be tested for the SNP to further inform their clinical management. However, herein lies the
266 next problem as there are few clinical alternatives to treating rUTIs other than advice and antibiotics.
267 Topical estrogen treatment is an option in reducing UPEC colonisation of the vaginal tissues and
268 helping to protect against repeated infections. Our clinical data, which showed elevated vaginal BD2
269 concentrations in patients prescribed estrogen treatment corroborated a previous report where
270 estrogen therapy enhanced the bladder innate response and strengthened the integrity of the
271 urothelium³². Our premise that estrogen functions synergistically to boost the urogenital innate
272 defences was further supported by our observation of a significant reduction in the urine and vaginal
273 douche BD2 concentrations of postmenopausal patients suffering rUTI, but carrying wild-type TLR5.
274 The report that vaginal estrogen is also associated with increased *Lactobacilli* growth, which impacts
275 on the survivability of uropathogens³⁶, further supports its use as a rUTI therapy. Yet, despite its
276 potential, vaginal estrogen is clinically acceptable only in post-menopausal women and due to
277 concerns including endometrial hyperplasia³⁷ is only recommended for limited periods. However,
278 other potential agents for therapeutic consideration-include hyaluronic acid and chondroitin sulphate,

279 whose intravesical use have been linked to decreased UTI recurrence and *in vitro* to host defence
280 peptide stimulation, including increased defensin synthesis³⁸⁻⁴⁰.

281 Our vaginal therapy option to help treat rUTI sufferers carrying the TLR5 SNP (Fig 5g) is based on the
282 premise that recurrence links to infections that are propagated through bacteria originating in the gut-
283 faecal material ascending the urethra into the bladder^{23,41,42}. However, recurrent infections can also be
284 explained using the intracellular bacterial community/quiescent intracellular reservoir (IBC/QIR) model
285 established through studies in mice⁴³. This model relies on UPEC ascending the urethra, invading the
286 bladder epithelium and forming either IBCs that contain metabolically-active bacteria capable of
287 infecting adjacent cells or non-replicating quiescent intracellular reservoirs. Following a stimulus,
288 possibly months after the initial infection and potentially involving urothelial turnover involving actin
289 rearrangements, these latent bacteria emerge to initiate a new acute infection. However, while the
290 IBC/QIR model has been demonstrated in multiple mouse backgrounds⁴⁴ there is limited evidence to
291 support its functioning in humans^{45,46}. Furthermore, the infection model does not fit well with data that
292 links vaginal treatments to reduced UTI incidence^{31,33}

293 The answer to developing rUTI therapies applicable for all groups of women lies in understanding the
294 disease pathology in relation to both the host response and bacterial virulence. To date, studies
295 investigating UTIs and potential new UTI treatments have focussed largely on the virulence factors
296 used by uropathogens at the primary site of infection, the bladder⁴³. While such information is not
297 generally taken into account in clinical decisions involving antibiotics, it has informed potential
298 treatment strategies including vaccination. However, the use of vaccines to treat rUTIs remains
299 tentative with whole pili immunogens proving ineffective and other methods focussed on UPEC toxins,
300 siderophores and the FimC-FimH complex, either providing no protection or reducing, but not totally
301 inhibiting bladder colonisation^{47,48}. Oral therapeutics called mannosides, which function as FimH
302 antagonists and reduce bacterial attachment at the bladder tissues show strong potential with the
303 efficacy of a new class, the C-mannosides, demonstrated *in vivo* using animal models of UTI^{49,50}.

304 Recently the FimH antagonist M4284 has been shown to reduce UPEC colonisation of the mouse gut
305 without impacting on the gut microbiota, and hence if developed therapeutically, could reduce UTIs
306 and rUTIs⁵¹. Deliberately establishing asymptomatic bacteruria in UTI-prone patients through the use
307 of non-adhering *E.coli*, has also been reported to be relatively successful in reducing UTI episodes⁵².

308 However, from our knowledge and understanding of the host innate defences we also advocate the
309 development and use of topical vaginal treatments that function, either by mimicking the action of

310 estrogen or through novel signalling pathways, to boost the endogenous innate defences of the
311 vaginal tissues and reduce vaginal *E.coli* colonisation (**Fig 5g**). We propose that such treatments, by
312 interrupting the UPEC infection cycle, will offer an additional, but simple antibiotic-sparing therapeutic
313 approach to the treatment of patients suffering rUTIs including those carrying the TLR5^{392Stop} SNP.

314

315

316 **Methods**

317 **Cell Culture**

318 The RT4 urothelial cell line (ATCC HTB-2)⁵³ was maintained without antibiotics in 25mM HEPES (4-
319 [2-hydroxyethyl]-1-piperazineethanesulfonic acid) modified RPMI 1640 medium supplemented with 2
320 mM glutamine and 10% fetal bovine serum (Sigma, Dorset, UK). VK2 E6/E7 cells (ATCC CRL-2616)
321 ⁵⁴ were maintained without antibiotics in keratinocyte serum-free medium (GIBCO, Paisley, UK)
322 containing 0.4mM calcium with 0.1 ng/ml human recombinant Epidermal Growth Factor (EGF) and
323 0.05 mg/ml bovine pituitary extract supplements. Normal human urothelial cells were isolated,
324 expanded and maintained in culture as previously described⁵⁵.

325 **Cell Challenge Studies**

326 RT4 and VK2 E6/E7 cells were seeded at 10⁴ cells/well into 30 mm, six well plates and cultured to
327 confluency at 37°C in 5% CO₂. Prior to challenge, cell monolayers were washed in phosphate
328 buffered saline (PBS) and incubated for 24h in fresh medium.

329 NU14⁵⁶, NCTC 10418 (ATCC 10536), K12⁵⁷ and CFT073⁵⁸ bacterial cultures were tested for motility
330 as described previously⁵⁹. Bacterial cultures were prepared for use in cell challenge experiments as
331 follows: Fifty µl of a 5ml log phase culture was used to inoculate a further 5 ml of medium and cultured
332 again to log-phase (3 h). Twenty µl of this culture was re-suspended in 980 µl PBS to give a working
333 suspension and 20 µl of this (approximately 5x10⁴ colony forming units (CFU)) used to inoculate each
334 well of eukaryote cells for the time required. Dead bacteria were produced by incubating live bacterial
335 stock solution for 30 minutes at 65 °C and confirmed by overnight agar plating. For bacterial
336 component challenges, each well of eukaryote cells was inoculated with 20 µL of either LPS,
337 Peptidoglycan (Invivogen, San Diego, California, USA) or *E. coli* flagellin⁶⁰. For BD2 enhancement
338 studies cells were supplemented with 4nM cyclodextrin encapsulated 17β-oestradiol dissolved in
339 water (Sigma) or 15nM (2-hydroxypropyl)-β-cyclodextrin for up to seven days prior to challenge. Once

340 the cells reached 80% confluency the cell culture medium was removed, the cells were washed with
341 PBS and incubated a further 24 hours in medium supplemented with 15nM cyclodextrin only. At each
342 appropriate challenge time points the medium was removed from the wells and stored at -20°C for
343 ELISA and the cells subjected to RNA extraction.

344 The time-kill antimicrobial assays using recombinant peptides, BD2 (Preprotech), LCN2 (Biovision),
345 and SLPI (RD systems) were adapted from the technique used previously⁶¹.

346 **NFκB Reporter Measurements**

347 RT4 cells stably transfected with a NFκB luciferase reporter⁶² and maintained under G418 selection
348 (Sigma, UK) at 0.5mg/ml were seeded onto 30 mm diameter, six well plates at a density of 10⁵
349 cells/well and cultured until 90% confluent. Following challenge with either PBS, bacteria or bacterial
350 components, the cells were lysed in RLB (Promega, Southampton, UK) for 16 hours at -80 °C and
351 following the addition of luciferin (Promega), luminescence was measured using a FluoStar Omega
352 microplate reader (BMG Labtech, Ortenberg, Germany). The results were presented as fold increase
353 over the control PBS challenge.

354 Activation of TLR-5 was inhibited by incubating cells with up to 5 µg/ml of mouse monoclonal TLR-5
355 antibody (maba2-htlr5, Invivogen, San Diego, USA) or IgG (5µg/ml) 2 hours prior to bacterial or
356 flagellin challenge.

357 **End-point, Quantitative RT-PCR and siRNA analyses**

358 Extraction of RNA from either cultured cells or the human tissue samples was performed using
359 TRIzol® reagent following the manufacturer's instructions (Invitrogen) and all RNA samples were
360 stored in RNAsin™ (Promega) at -80 °C. cDNA preparation and transcript abundance, measured by
361 either end-point or quantitative RT-PCR, was as previously described⁶¹. Quality of extracted RNA
362 was assessed using a Bioanalyzer 2100 (Agilent Technologies, Berkshire, UK) to ensure a minimum
363 RIN of 8.0 for quantitative real time PCR (RT-qPCR) analyses of the *in vitro* samples and 7.0 for
364 biopsy samples. Primers and annealing temperatures are presented in Table 1.

365 Transfection of VK2 cells with TLR5 siRNA (s14199:ThermoFisher) was performed using Viromer
366 Green (Lipocalyx) following the manufacturer's protocol. The 48h knockdown was followed by a 24h
367 flagellin challenge.

368

369 **Bacterial Growth Measurements**

370 The immunosorbent experiments utilised urines collected from both flagellin and CFT073 challenged
371 C57BL/6 wild-type mice. Urine samples were added to BD2 or Avian BD9 antibody (Cambridge
372 Research Biochemicals, Cleveland, U.K.) coated ELISA plates (Leinco Technologies) and following a
373 2h incubation the supernatants recovered and stored at -20 °C. Supernatants (100 µl/well) were used
374 in bacterial growth experiments performed in 96 well microtitre plates and data analysed in relation to
375 lag time extension⁶³. For bacterial growth, over-night cultures of CFT073 were inoculated into Luria
376 broth, grown to mid-log phase (OD₆₀₀=0.4), diluted to 10⁴ cells/ml and aliquoted into wells of 96-well
377 microtitre plates containing control or biological samples. The plates were incubated for 16 h in a
378 Fluostar Omega plate reader set at 37°C, shaken at 200rpm every 20 min and the bacterial OD₆₀₀
379 measured every 20 min.

380 **Human Beta-Defensin 2 (BD2) and Interleukin 8 (IL-8) Measurements**

381 Human BD2 was quantitated in clinical and cell culture samples using a Human BD2 ELISA
382 Development Kit (Leinco Technologies, St Louis, Missouri, USA). IL-8 concentrations were analysed
383 using the BD optEIA IL-8 ELISA kit (BD Bioscience, Franklin Lakes, USA).

384 **Mice Experiments**

385 C57BL/6J wild-type and B6.129S1-Tlr5^{tm/Flv}/J (Tlr5^{-/-} knockout) female mice (obtained from Jackson
386 Laboratories, Bar Harbor, ME.) were bred to between 6 and 8 weeks of age at the Duke University
387 Medical Center animal care facility. *E. coli* CFT073 and *E.coli* flagellin were used for all murine
388 infections. CFT073 cultures were inoculated from frozen stock into Luria broth [Becton Dickinson and
389 Company (BD), Franklin Lakes, NJ] and grown overnight at 37°C. Optical density (OD) was
390 determined and cultures were washed, and diluted in PBS. Bladder infections were performed as
391 described previously⁶. Essentially mice were anesthetized, catheterized with polyethylene tubing
392 (inner diameter: 0.28 mm) (BD), and 50 µl of PBS (control) or PBS containing either 10⁸ bacteria or 5
393 µg *E.coli* flagellin instilled from a 1 ml tuberculin syringe with a 30G1/2 needle. Mice were euthanized
394 by CO₂ asphyxiation and whole tissues (vaginas, bladders and kidneys) isolated for transcript
395 analyses and bacterial CFU quantification. All animal experiments were approved by the Duke
396 University IACUC and Division of Laboratory Animal Resources, and performed in accordance with
397 relevant guidelines and regulations.

398 **Human Subjects**

399 The study was approved by the County Durham & Tees Valley 1 NHS Research Ethics Committee
400 (09/H0905/15) and Newcastle upon Tyne Hospitals NHS Trust (ID 4841). Written informed consent
401 was obtained from all participants and all methods were performed in accordance with relevant
402 guidelines and regulations. Women with structurally-normal urinary tracts and consenting to provide
403 biopsies, samples of blood, urine and vaginal washings were recruited from the Urology and Uro-
404 gynaecology departments of Newcastle upon Tyne Hospitals NHS Trust from July 2009 to February
405 2011. Controls were recruited from women attending for investigation of haematuria or other non-
406 infection related uro-gynaecological assessment; rUTI sufferers were women who had suffered either
407 at least two episodes per year for two years or three episodes in the previous year. Other inclusion
408 criteria required subjects to be aged 18 years or older; pre-menopausal or at least six months
409 post-menopause; no antibiotic therapy within four weeks of recruitment. Pre-investigation sample size
410 calculations based on previous reported urinary AMP concentrations indicated that 18 women in each
411 of the four groups (72 in total) would give 80% chance of detecting a difference at 5% significance
412 level. The women were stratified according to menopausal status to create four groups: 19 Pre-
413 menopausal controls (median age 35: range 18-46); 31 Pre-menopausal cases (rUTI sufferers)
414 (median age 31: range 18-41) for which there were 26 complete analyses; 19 Post-menopausal
415 controls (median age 58: range 42-76); 29 Post-menopausal cases (rUTI sufferers) (median age 60:
416 range 40-75) for which there were 22 complete analyses. Clinical histories of rUTI and control
417 subjects are presented in Table 2.

418 **Patient samples**

419 Biopsies from the first 2-3 cm of the vagina and posterior bladder wall were obtained during
420 cystoscopy. Following cystoscopy, the first vaginal washing or douche was collected using the
421 'Summer's Eve Cleansing Douche' (Fleet Laboratories, Lynchburg, Virginia, USA) according to the
422 manufacturer's instructions. Each subject was given the necessary instructions and containers to
423 collect an overnight urine specimen (approximately 600-800ml) and a further vaginal douche 6-8
424 weeks later. Pre-menopausal subjects were requested to collect the douche at the mid-point of their
425 menstrual cycle. Of the subjects that gave two douche samples, all were post-menopausal; nine were
426 rUTI sufferers prescribed vaginal estrogen (Vagifem twice weekly 10mcg) and 12 were rUTI sufferers
427 given treatments including antibiotic prophylaxis and/or advice (one did not complete).

428 **Targeted TLR5 Sequencing**

429 Blood samples taken from each subject were processed for genomic DNA (gDNA) extraction using
430 the GeneCatcher™ gDNA 3–10 mL Blood Kit (Invitrogen, Paisley, UK). PCR amplification of the
431 TLR5^{392Stop} containing region of the TLR5 gene was carried out on 100ng of gDNA using Novagen
432 Kod Host start DNA Polymerase (Merck Millipore, Darmstadt, Germany) and the TLR5 Primer pair
433 (forward primer, GGTAGCCTACATTGATTTGC; reverse primer,
434 GAGAATCTGGAGATGAGGTACCCG). A 10µL aliquot of the 461 bp product was used for
435 sequencing (Genevision, Newcastle upon Tyne, UK). Sequencing data was provided in AB1 format
436 and data analyses performed using the freeware FinchTV trace viewing software (Geospiza, Seattle,
437 USA).

438 **Immunohistochemistry**

439 Vaginal samples for immunohistochemistry were fixed in formalin overnight before being transferred
440 to 70% ethanol for long term storage or paraffin embedding. Sections of 5-µm thickness were
441 mounted on microscope slides and following microwave-heat mediated antigen retrieval were stained
442 with anti-beta 2 Defensin primary antibody (Abcam, ab63982)1/1000 (1 X PBS), overnight at 4°C,
443 followed by one hour at room temperature with polyclonal goat anti-rabbit Immunoglobulins/HRP
444 1/3000 (DAKO). Images were taken using a Nikon Eclipse Ti, coupled to a Photometrics Coolsnap
445 HQ CCD camera and Nikon Plan Fluor 100x/1.30 ph3 DL lens.

446

447 **Statistical Analyses**

448 All statistical analyses were performed out using the Prism 5 Software package (GraphPad Software
449 Inc, La Jolla, California, USA). Significance of data with two groups was determined by unpaired two-
450 tailed Student's t test; for more than two groups a two-way analyses of variance followed by Tukey's
451 multiple comparison or Bonferroni post-tests, as appropriate, was used. For comparison of the first
452 and second douche data, a paired two-tailed test was utilised.

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Figure legends

Figure 1: VK2 E6/E7 and RT4 *DEFB4* and BD2 responses to UPEC and flagellin challenges.

End point PCR panels of host defence peptide gene expression in VK2 E6/E7 and RT4 cells following 24hr challenge with PBS (control), flagellin (250ng/ml) or UPEC (10^5) (1a). Full length gels are shown in Supplementary File 1 (S1a-d). *DEFB4* transcript expression in VK2 E6/E7 and RT4 cells following challenges with flagellin (250ng/ml) and heat-killed flagellated (NCTC10418) or non-flagellated (NU14) *E.coli* (10^5) (N=3, n=9) (1b). BD2 concentrations measured in VK2 E6/E7 and RT4 cell media following 24 hour challenges with flagellin (250ng/ml) and heat-killed CFT073 (10^5) (N=3, n=6) (1c). BD2 peptide concentrations of primary ureteric urothelial cells challenged with *E.coli* flagellin (250ng/ml) for 24 hours (N=3, n=6) (1d). *DEFB4* transcript expression, presented as relative expression, of primary vaginal epithelial cells challenged with *E.coli* flagellin (50ng/ml) for 24 hours (N=6) (1e). *E.coli* swarming motility on LB agar media; BD2 peptide concentrations measured in VK2 E6/E7 and RT4 cell media following 24 hour challenges with flagellin (250ng/ml) and heat-killed bacteria (10^5) (N=3, n=6) (1f). *DEFB4* transcript expression of VK2 E6/E7 cells transfected with either negative siRNA or TLR5 siRNA and challenged for 24 hours with flagellin (50ng/ml) (N=3) (1g). BD2 peptide concentrations of VK2 E6/E7 cells incubated with TLR5 blocking antibody (1-5ug/ml) or IgG (5ug/ml) and challenged 24 hours with flagellin (250ng/ml) (N=3) (1h). Fold change in NF κ B-luciferase activity in RT4 cells challenged with 250ng/ml flagellin (FLG) for 2-8 hours, peptidoglycan (PG) (10 μ g/ml) for up to 16 hours and Lipopolysaccharide (LPS) (10 μ g/ml) for up to 16 hours (N=3, n=6 (FLG), n=9 (PG & LPS)) (1i). All data presented as mean \pm SEM, * P<0.05, ** P<0.01, *** P<0.001.

Figure 2: *DefB4* expression in mouse urogenital tissues following UPEC and flagellin challenge

DefB4 mRNA expression in wild type C57BL6 and *Tlr5*^{-/-} mice bladders following 6h challenge with either flagellin (5 μ g) or CFT073 (10^8) (N=3) (2a). Vaginal *DefB4* mRNA expression in wild type C57BL6 and *Tlr5*^{-/-} mice following 6h challenge with either flagellin (5 μ g) or CFT073 (10^8) (N=3) (2b). *E. coli* colonisation of the bladders and kidneys of wild type C57BL6 and *Tlr5*^{-/-} mice following 6h CFT073 bladder challenge (N=3) (2c). All data presented as mean \pm SEM, * P<0.05, ** P<0.01.

Figure 3: Effects of host defence peptides on CFT073 growth

Time-kill data showing CFT073 survival following two hour incubation with host defence peptides BD2 (30 and 300ng/ml: 7.5nM and 75nM), LCN2 (300 ng/ml: 13nm) and SLPI (300 and 3000ng/ml: 25 and 250nM) (N=4). Bacterial growth values >100% link to peptide concentrations not associated with bacterial killing suggesting amino acids associated with peptide degradation, either natural or bacterial protease activity, are used by the bacteria as a growth source (3a). CFT073 (10^4) growth times in wild type mice urines depleted of DefB4 (controls: no depletion and Avian defensin antibody-specific depletion) (3b). All data presented as mean \pm SEM, * P<0.05, *** P<0.001.

Figure 4: *DEFB4* and *TLR5* mRNA expression, and BD2 responses in control and rUTI patient samples

DEFB4 mRNA expression, presented as relative expression (4a) and BD2 peptide concentrations (4b) in vaginal biopsies from control (N=38) and rUTI patients (N=48). Vaginal douche data stratified to show BD2 peptide concentrations of premenopausal (Pre-M) control subjects (N=19), premenopausal rUTI patients (N=26), postmenopausal (Post-M) control subjects (N=19) and postmenopausal rUTI patients (N=22) (4c). *TLR5* mRNA expression, presented as relative expression, in vaginal biopsies from control subjects, patients with rUTI and rUTI at time of an active infection (4d). Urine BD2 concentrations of the rUTI No SNP (N=8) and *TLR5*^{392Stop} SNP patients (N=5) and vaginal douche BD2 peptide concentrations N=9 (No SNP) and N=5 (SNP) during periods of active infection (4e). Matched urine BD2 concentrations of rUTI non SNP (N=8) and *TLR5*^{392Stop} SNP patients (N=5) analysed during periods of infection and non-infection (4f). Vaginal douche BD2 peptide concentrations of no SNP rUTI patients measured during an acute infection alongside vaginal douche BD2 concentrations of the non-infected pre-menopausal control subjects (4g).

NF κ B-luciferase activity (fold change) in RT4 cells following 2 to 24 hour challenges with heat killed UPEC (5×10^4) isolated from the urines of three rUTI SNP patients during an acute infection (4h). NF κ B-luciferase activity (fold change) in RT4 cells incubated in presence or absence of *TLR5* blocking antibody (5ug/ml) or IgG (5ug/ml) following 24 hour challenge with flagellin (250ng/ml) or heat killed UPEC isolated from each of the three SNP patients (4i). IL-8 concentrations (pg/ml) in media of RT4 cells incubated in presence or absence of *TLR5* blocking antibody (5ug/ml) and challenged for 24 hours with flagellin (250ng/ml) and bacteria isolated from each of the three SNP

patients (4j). BD2 concentrations (pg/ml) in media of RT4 bladder cells incubated in presence or absence of TLR5 blocking antibody (5ug/ml) and challenged for 24 hours with either flagellin (250ng/ml) or bacteria isolated from each of the three SNP patients (4k). N=2, n=6 and all data presented as mean \pm SEM, * P<0.05, ** P<0.01, *** P<0.001.

Figure 5: Effects of estrogen treatment on BD2 responses *in vitro* and rUTI patients

VK2 E6/E7 cell *DEFB4* mRNA expression following estrogen (4nM) seven day pretreatment and 24h challenge with either cyclodextrin (15nM), flagellin (50ng/ml), or flagellin (50ng/ml) and estrogen (4nM) N=3 (5a). BD2 peptide concentrations measured in the VK2 E6/E7 cell media following estrogen (4nM) seven day pretreatment and either 24h or 48h challenge with either cyclodextrin (15nM), estrogen (4nM), flagellin (50ng/ml) or flagellin and estrogen (N=3) (5b). Vaginal douche BD2 peptide concentrations of rUTI patients before and after 6-8 weeks of treatment with antibiotic prophylaxis and/or advice (N=12) (5c), or 6 to 8 weeks treatment with vaginal estrogen (Vagifem, 10mcg, twice weekly) (N=9) (5d). Staining of vaginal tissue from estrogen treated (+E) and control subjects for immunoreactive BD2 (5e). Vaginal douche BD2 peptide concentrations of rUTI TLR5 SNP patients before and after treatment (6 to 8 weeks) with vaginal estrogen (N=3) or antibiotic prophylaxis (N=1) (5f). All data presented as mean \pm SEM, ** P<0.01, *** P<0.001.

Schematic illustrating (i) how an inherited or acquired reduction in the vaginal host innate defences can facilitate an UPEC infection of the urogenital tract and (ii) how vaginal treatments enhancing the host innate defences can help prevent this (5g). 1. Uropathogenic *E.coli* (UPEC) originating from bowel migrate from rectum towards vagina; 2. Inherited or acquired reduction in innate defences including HDP and chemokines facilitates increased vaginal and peri-urethral colonisation by UPEC; 3. Urethral ascent of UPEC from peri-urethral and vaginal regions to bladder; 4. Bladder infection; 5. Vaginal topical agent stimulates host innate defences and UPEC destroyed preventing vaginal colonisation; 6/7. Urethra and bladder protected from UPEC infection.

Table 1: Primer sequences

Gene	Primer Sequence	Primer Type	T _m (°C)
<i>DEFB1</i>	Reverse: <u>cgcc</u> GGTAGGAAGTTCTCATGGcG Forward: GTCAGCTCAGCCTCCAAAGGA	Probes Master	60
<i>Defb4</i>	Forward: GTGAAGCTCCCAGCCATCAG Reverse: GATTGCGTATCTTTGGACACC	SYBR Green I Master	58
<i>DEFB4</i>	SA Biosciences commercial primer	SYBR Green I Master	60
<i>DEFA5</i>	Forward: GCCATCCTTGCTGCCATTC Reverse: GATTTACACACCCCGGAGA	SYBR Green I Master	58
<i>hCAP-18</i>	Forward: <u>cgct</u> GACGGGCTGGTGAAGcG Reverse: CCCAGCAGGGCAAATCTCTT	Probes Master	55
<i>TLR5</i>	Forward: CAGAGACTGGTGTCAAGGAC Reverse: GTGTCCAGGTGTTGAGCA	SYBR Green I Master	54
<i>GAPDH</i>	Primer Design commercial primer (House-keeping reference gene)	SYBR Green I Master	60
<i>ATP5B</i>	Primer Design commercial primer (House-keeping reference gene)	SYBR Green I Master	60
Mouse β -actin	Forward: TGAGAGGAAATCGTGCGTGACAT Reverse: ACCGCTCATTGACGATAGTGATGA	SYBR Green I Master	60

	Control		rUTI	
	Pre-menopause	Post-menopause	Pre-menopause	Post-menopause
Numbers Recruited	19	19	31	29
Median Age (Range)	35 (18-46)	58 (42-76)	31 (18-41)	60(40-75)
Prescribed vaginal estrogen previously	1	2	0	0
Taking vitamins	4	3	4	4
Taking cranberry-juice	5	5	16	15
On oral contraception	6	0	4	0
Had HRT	0	4	0	9

Table 2: Clinical History









