Title: Optimization of an Experimental Vaccine to Prevent *Escherichia coli* Urinary Tract Infection

Running Title: Optimization of a UPEC vaccine

Authors: Valerie S. Forsyth[a], Stephanie D. Himpsl[a], Sara N. Smith[a], Christina A. Sarkissian[a*], Laura A. Mike[a], Jolie A. Stocki[a], Anna Sintsova[a*], Christopher J. Alteri[a,b], Harry L.T. Mobley[a#]

Author Affiliations:

[a]Department of Microbiology and Immunology; University of Michigan Medical School; Ann Arbor, MI, 48109; United States

[b]Department of Natural Sciences; University of Michigan Dearborn; Dearborn, MI, 48128; United Sates

[#]**Corresponding Author:**

hmobley@umich.edu (HLTM)

*Present address:

Christina A. Sarkissian, Arbor Biosciences, Ann Arbor, MI, USA.

Anna Sintsova, University of Zurich, Zurich, Germany.

Abstract word count: 250

Text word count: 4,953

Abstract (250 words)

Urinary tract infections (UTI) affect half of all women at least once during their lifetime. The rise in extended-spectrum beta-lactamase-producing strains and potential for carbapenem resistance within uropathogenic *Escherichia coli* (UPEC), the most common causative agent of UTIs, creates an urgent need for vaccine development. Intranasal immunization of mice with UPEC outer membrane iron receptors, FyuA, Hma, IreA, or IutA, conjugated to cholera toxin, provides protection in the bladder or kidneys when challenged with UPEC CFT073 or 536. Based on these data, we sought to optimize the vaccination route (intramuscular, intranasal, or subcutaneous) in combination with adjuvants suitable for human use including alum, monophosphoryl lipid A (MPLA), unmethylated CpG synthetic oligodeoxynucleotides (CpG), polyinosinic:polycytodylic acid (polyIC), and mutated heat-labile *E. coli* enterotoxin (dmLT). Mice intranasally vaccinated with dmLT-IutA or dmLT-Hma displayed a significant reduction in bladder colonization (86-fold and 32-fold, respectively) with 40–42% of mice having no detectable colony forming units (CFU). Intranasal vaccination of mice with CpG-IutA and polyIC-IutA significantly reduced kidney colonization (131-fold) and urine CFU (22-fold), respectively. dmLT generated the most consistently robust antibody response in intranasally immunized mice, while MPLA and alum produced greater concentrations of antigen-specific serum IgG with intramuscular immunization. Based on these results, we conclude that intranasal administration of Hma or IutA formulated with dmLT adjuvant provides the greatest protection from UPEC UTI. This study advances our progress toward a vaccine against uncomplicated UTI, which will significantly improve the quality of life for women burdened by recurrent UTI and enable better antibiotic stewardship.

53 ## Importance (105/150 words)

54 Urinary tract infections (UTI) are among the most common bacterial infection in humans,
55 affecting half of all women at least once during their lifetimes. The rise in antibiotic resistance
56 and health care costs emphasizes the need to develop a vaccine against the most common UTI
57 pathogen, *Escherichia coli.* Vaccinating mice intranasally with a detoxified heat-labile
58 enterotoxin and two surface exposed receptors, Hma or IutA, significantly reduced bacterial
59 burden in the bladder. This work highlights progress in the development of a UTI vaccine
60 formulated with adjuvants suitable for human use and antigens that encode outer membrane iron
61 receptors required for infection in the iron-limited urinary tract.

Introduction

Urinary tract infections (UTI), the second most common human infection after respiratory infections, result in an annual cost of $3.5 billion (1, 2). Uropathogenic *Escherichia coli* (UPEC) is the most prevalent causative agent of uncomplicated UTI, rates of antibiotic resistance in pathogenic isolates are increasing, and multidrug resistant strains (*E. coli* ST131) are emerging (1-3). Despite innate immune defenses in the bladder that include micturition, a mucin layer, constitutively expressed secretory immunoglobulin A, cationic antimicrobial peptides, Tamm-Horsfall protein, lactoferrin, and lipocalin-2 (4), half of all women will experience a UTI in their lifetime with 1 in 40 women experiencing recurrent infections (5). Patients with acute or recurrent UTI have significantly decreased levels of total secretory IgA in the urine as compared to healthy individuals with no history of UTI (6, 7). This indicates the potential for decreased severity and duration of infection if microbe-specific antibody levels can be increased with a vaccine. Because 90% of symptomatic UTI are uncomplicated infections, an ideal vaccine will target factors critical for establishment of bladder colonization (3) Five FDA-approved vaccines provide mucosal protection against other pathogens including poliovirus, rotavirus, influenza virus, *Salmonella enterica* serovar Typhi, and *Vibrio cholerae* (8-11). These efficacious mucosal vaccines that protect against other enteric viruses and bacteria bolster the hypothesis that a vaccine effective against uropathogens is attainable.

During the last 20 years there have been noteworthy advancements toward the development of a UTI vaccine, yet no licensed UTI vaccines are available for use in the U.S. Published studies have investigated the efficacy of vaccines containing O antigen (12), fimbrial subunits (13, 14), α-hemolysin (15), siderophores (16), and a variety of outer membrane siderophore receptors in animal models of UTI (17-20). Human clinical trials have been

85 performed on three vaccines, Uro-Vaxom, SolcoUrovac, and ExPEC4V. Uro-Vaxom,
86 comprised of 18 *E. coli* uropathogen extracts and administered as a daily oral tablet, is approved
87 in Germany and Switzerland for the prevention of recurrent cystitis (21). SolcoUrovac, currently
88 marketed as StroVac, contains heat-killed uropathogenic bacteria including *E. coli*, *Proteus*
89 *vulgaris*, *Klebsiella pneumoniae*, *Morganella morganii*, and *Enterococcus faecalis* and is
90 approved for human use in Europe (3, 22). ExPEC4V consists of four conjugated O-antigens
91 O1A, O2, O6A, and O25B common to *E. coli* strains known to cause UTI (23). In a study
92 comparing the efficacy of these three vaccines in adults with recurrent UTIs, Uro-Vaxom had the
93 greatest reduction in rate of UTI recurrence while ExPEC4V did not appear to reduce UTI
94 recurrence (24). Nonetheless, the daily regimen and toxic side effects have limited widespread
95 use of Uro-Vaxom (25).

96 Here we describe our efforts to develop a vaccine against uncomplicated UTIs using
97 antigens previously identified and validated as vaccine candidates by intranasal immunization in
98 a murine UTI model when conjugated to the adjuvant cholera toxin (26-28). We previously
99 performed an extensive multi-omics approach to identify genes and their proteins that: 1) are
100 localized to the bacterial cell surface (29); 2) are expressed during growth in human urine (30),
101 murine infection (31), and human infection (32, 33); 3) possess immunoreactive properties (34);
102 and 4) are more prevalent in UPEC isolates than commensal isolates (35, 36). A total of four β-
103 barrel outer membrane receptors required for iron sequestration met all of these criteria including
104 heme receptor Hma, aerobactin receptor IutA, yersiniabactin receptor FyuA, and putative
105 siderophore receptor IreA. Effective iron acquisition from the iron-limited environment of the
106 urinary tract is required for full virulence of UPEC (37-39). In addition to their iron scavenging
107 function, IreA functions as an adhesin that is important for colonization of the bladder (38) and

FyuA plays a role in biofilm formation in human urine (40). Intranasal immunization with Hma, IreA, IutA, or FyuA, conjugated to cholera toxin, significantly reduced in bacterial burden in the bladder, kidneys or both 48 hours following transurethral challenge with UPEC (27, 28). While cholera toxin is an effective immune stimulant in mice, it is not suitable for human use, due to development of profuse diarrhea with oral doses as low as 5 µg (41). Because of this drawback, we sought to optimize this UTI vaccine by incorporating adjuvants approved for use in humans or used in vaccine clinical trials.

The precise immune response required for protection against UTI is not well-defined. Therefore, we selected a panel of adjuvants known to elicit an array of adaptive immune responses, with the aim being to identify an adjuvant that is well-suited for protecting against UTI and safe for use in humans (3, 42). The five adjuvants tested were alum (43, 44), monophosphoryl lipid A (MPLA) (45-47), unmethylated CpG synthetic oligodeoxynucleotides (CpG) (48-50), polyinosinic:polycytodylic acid (polyIC) (51), and double mutant (R192G/L211A) heat-labile *E. coli* enterotoxin (dmLT) (52). Alum is licensed for use in twenty-two vaccines available in the U.S. and is reported to activate dendritic cells via multiple mechanisms, thus promoting antigen uptake and release of IL-1β and IL-18 (43, 53). MPLA is derived from *Salmonella minnesota* R595 lipopolysaccharide, activates cellular immunity through the TLR4 signaling pathway, is approved for human use in Europe, and is a component of vaccines for hepatitis B and papilloma viruses (51, 54). CpG activates TLR9 signaling in B cells and dendritic cells, increases mucosal immune responses, and is licensed for use in a hepatitis B vaccine (46, 47, 55). Both dmLT and CpG are presumed to function by activating innate signaling and stimulation of mucosal dendritic cells, which activate the adaptive immune response, particularly Th17 cells by dmLT (52, 56-58). polyIC is a synthetically produced

131 double stranded RNA, analogous to viral RNA, that induces a robust type I interferon response
132 resulting in activation of cellular immunity, and is in late stage clinical development (51).

133 In an effort to develop a vaccine protective against uncomplicated UTI in humans, we
134 tested five adjuvants (dmLT, CpG, polyIC, MPLA, and alum) with four antigens (Hma, IreA,
135 IutA, or FyuA) for efficacy in mice. Because immunization route can affect the immune
136 response, we examined three routes of immunization: intranasal, intramuscular, and
137 subcutaneous with multiple antigen-adjuvant combinations. Hma and IreA, which have been
138 shown to significantly reducepreviously demonstrated to provide the most robust reduction of
139 bacterial burden in the kidneys and bladder (28), respectively, were initially examined under all
140 conditions, then the most promising combinations of route and adjuvant were further evaluated
141 for efficacy with the remaining two antigens FyuA and IutA. Here we report that intranasal
142 immunization with dmLT-Hma and dmLT-IutA induces antigen-specific antibody production
143 and provides robust protection in immunized mice following transurethral challenge with UPEC.

144

Results

Immunizing via the intranasal route provides the most protection against UTI. We previously established that intranasal immunization followed by two weekly boosts with outer membrane iron receptors Hma, IreA, IutA or FyuA conjugated to cholera toxin significantly reduced bacterial burden in the bladder, kidneys or both, 48 hours following transurethral challenge with UPEC (27, 28). We later determined that conjugation of antigen to adjuvant was not required for protection (unpublished results). Based on these data, we began systematic optimization of the vaccine to maximize efficacy using alternative adjuvants admixed with antigen. The optimized vaccine route, adjuvant and antigen were evaluated based on three criteria: reduction of bacterial CFU in the sample sites, increased number of mice without detectable bacterial counts, and production of antigen-specific antibody. Genes encoding all protein antigens were codon-optimized, proteins were purified from inclusion bodies and certified LPS-free.

The immunization route can markedly affect the efficacy of vaccines, and some routes are linked to greater patient compliance especially when immunization requires multiple boosts (59, 60). In an effort to determine the optimal route, we systematically immunized mice with one of five adjuvants either intranasally, intramuscularly, or subcutaneously in combination with either Hma or IreA, which have been shown to significantly reduce bacterial burden in the kidneys and bladder, respectively (28). Promising combinations of route and adjuvant were further evaluated for efficacy by imunizing with the remaining two antigens FyuA and IutA. Intramuscular and subcutaneous routes were chosen to expand upon early success with intranasal immunization because they are commonly used deliver vaccines in humans. Mice were

167 immunized as previously described (28). In total, 35 immunization trials utilizing 1,060 mice
168 were performed.

169 We found that intranasal immunization tended to reduce median bacterial burden at least
170 two-fold in the urine for all antigen-adjuvant combinations tested (Table 1). When immunized
171 intranasally, two-fold reduction in median bacterial burden in the bladder occurred for 58%
172 (7/12) of combinations tested and in the kidneys for 33% (4/12) of combinations tested (Table 1).
173 Significant differences are noted in bold in Table 1. Specifically, intranasal immunization with
174 polyIC-IutA (*P*=0.059 urine), dmLT-Hma (*P*=0.024 bladder), dmLT-IutA (*P*=0.018 bladder) and
175 CpG-IutA (*P*=0.046 kidneys) significantly reduced bacterial burden (Figure 1B, E, F, J, Table 1).
176 Three adjuvant-antigen combinations tended to reduce colony forming units (CFU) more than
177 two-fold in all sites of infection following intranasal immunization: dmLT-IutA, CpG- Hma, and
178 CpG-IreA (Table 1). The bimodal distribution of bladder colonization observed with protective
179 combinations (dmLT-Hma, dmLT-IutA) is typical of this model (19, 20).

180 Immunization via the intramuscular and subcutaneous routes showed limited protection.
181 Intramuscular immunization utilizing Hma as antigen tended to reduce median bacterial burden
182 at least two-fold in 3 out of 12 trials (Table 1). Immunization with IreA intramuscularly did not
183 reduce bacterial burden two-fold or greater in any trial. The subcutaneous route in combination
184 with Hma tended to reduce median CFU at least two-fold in the urine when formulated with
185 alum or dmLT, and in the kidneys when formulated with MPLA (Table 1). No reduction in
186 bacterial burden was observed for any of the sites of infection when any of the IreA vaccine
187 formulations were administered subcutaneously (Table 1).

188 Bacterial inocula for the thirty-five infection trials were confirmed to be at the desired
189 dose with the median dose at $3.05 \pm 0.11 \times 10^9$ CFU/mL of strain CFT073 and $2.70 \pm 0.20 \times 10^9$

CFU/mL of strain 536 (Supplemental Figure 1). Strain CFT073 lacks a functional FyuA, therefore vaccine combinations containing this antigen utilized *E. coli* strain 536 for challenge. These data verify that variances in colonization level between the experimental trials were not due to differences in bacterial dose used to transurethrally inoculate mice (Supplemental Figure 1). When polyIC or CpG was administered intranasally in the absence of antigen, the median colonization level of CFT073 was not significantly different from that of unimmunized mice infected with the same dose of CFT073 (Supplemental Figure 2), indicating that polyIC or CpG alone do not alter colonization of CFT073. However, intranasal administration of dmLT as adjuvant in the absence of antigen significantly reduced the bacterial burden in the kidneys (P=0.02) (Supplemental Figure 2), consistent with previous findings that dmLT alone reduced colonization of multiple pathogens including *Haemophilis influenzae*, *Campylobacter jejuni*, and *Shigella flexneri* (52).

Taken together these results indicate that intranasal immunization tended to improve the protective response in the urine, bladder and kidneys at least two fold following bacterial challenge for 64% (23/36) of the adjuvant-antigen combinations, five of these were statistically significant, in comparison to intramuscular immunization 33% (8/24, P = 0.03) or subcutaneous immunization 13% (3/24, $P < 0.005$) (Table 1). No statistically significant CFU reductions were observed in any combination administered by the intramuscular or subcutaneous routes. Therefore, intranasal immunization is the optimal route for protection against transurethral challenge with *E. coli*.

dmLT is the adjuvant that provides the most effective protection against colonization by *E. coli*. Having determined that intranasally administering different vaccine formulations provides the most consistent protection against UTI, we next set out to optimize the

adjuvant. Previous studies have shown that immunizing with cholera toxin conjugated to antigens provides a robust immune response and reduces bacterial burden in the bladder and kidneys of CBA/J mice (27, 28), however, it is not suitable for human use (41). In an effort to develop a vaccine to prevent UTI, we tested our antigen candidates admixed with alternative adjuvants approved for human immunization: dmLT, CpG, polyIC, MPLA, and alum, for efficacy in mice. Intranasal immunization with dmLT, an adjuvant very similar in structure to cholera toxin (both are A_1B_5 toxins), significantly reduced the median bladder colonization (P = 0.02) when admixed with the antigen Hma compared to dmLT alone (Figure 1E). In addition, this vaccine combination significantly increased the number of mice without detectable bacteria in the bladder (35% dmLT only, 68% dmLT-Hma, P = 0.056), and urine (26% dmLT only, 61% dmLT-Hma, P = 0.05) indicating that more mice cleared the infection (below the limit of detection) 48 hours post-inoculation (Supplemental Table 1). Similar to Hma, immunizing with dmLT-IutA significantly reduced the median CFU/g bladder (P = 0.02) (Figure 1F) and significantly increased the number of protected mice (20% dmLT only, 67% dmLT-IutA, P = 0.03) (Supplemental Table 1). In addition, dmLT administered with FyuA and IutA tended to reduce urine bacterial load two-fold (Table 1).

Other adjuvant-antige combinations that resulted in significant reductions in bacterial burden when administered intranasally include polyIC-IutA (P = 0.05 urine) (Figure 1B) and CpG-IutA (P = 0.05 kidneys) (Figure 1J). The primary target of this vaccine is patients with uncomplicated UTI, therefore, reducing bladder colonization and inflammation (cystitis) is the desired outcome. Since dmLT-IutA and dmLT-Hma both reduced bacterial burdens in the bladder, dmLT was selected for future studies.

IutA is the optimal antigen when co-administered with dmLT via the intranasal route. Previous studies with cholera toxin conjugated to antigens found that IreA and IutA provided significant protection from bacterial challenge in the bladder and Hma, FyuA, and IutA provided protection in the kidneys (27, 28). When administered via the intranasal route and formulated with dmLT, individually all four antigens trended toward reducing bacterial burdens in the urine (Figure 1A-D). In the bladder, CFU/g tissue was significantly reduced compared to adjuvant alone when Hma (32 fold-reduction, P=0.024) and IutA (86 fold-reduction, P=0.018) were co-administered with dmLT (Figure 1E, F). FyuA and IreA did not provide any protection compared to the adjuvant alone cohort (Table 1, Figure 1G, H). IutA was the only antigen that reduced colonization more than two-fold in the kidneys, although this reduction was not statistically significant (Table 1). According to these results, IutA is an effective antigen for reduction of bacterial burden when administered via the intranasal route in combination with dmLT in all sites of infection. In comparison Hma is an effective antigen in two sites of infection.

Antigen-specific antibody response to immunization. In addition to evaluating the vaccine efficacy by observing changes in the bacterial burden after challenge, the humoral response was evaluated using an indirect ELISA. The amount of antigen-specific IgG in serum collected one week after the second boost was quantified. In all trials, independent of route, adjuvant or antigen, mice immunized with adjuvant alone had no measurable amounts of antigen-specific antibody (Figure 2, 3 Supplemental Figure 3). However, mice immunized with adjuvant admixed with antigen elicited a statistically significant increase in the concentration of antigen-specific IgG in the serum compared to controls immunized with adjuvant alone (P = 0.05) (Figure 2, 3 Supplemental Figure 3). This indicated that addition of adjuvant alone does

not produce an antigen-specific immune response, and outer-membrane iron receptor preparations containing no LPS contamination are antigenic.

The immune responses generated between routes of immunization and adjuvants varied. Intranasal immunization with dmLT generated the most consistent and robust serum antibody response across all antigens, with median concentration of antigen-specific IgG 12.0 µg/mL Hma, 21.6 µg/mL IreA, 5.8 µg/mL FyuA, 22.4 µg/mL IutA (Figure 2A). When compared to vaccine formulations that significantly reduced bacterial burden in the bladder of challenged mice, there was no correlation between antigen-specific antibody production and protection for individual mice, indicating that the mechanism of protection may include cell-mediated immunity (Supplemental Figure 4). In comparison to immunizations with dmLT, polyIC in combination with all antigens produced a weak antigen-specific antibody response with an average concentration of 3.2 µg/mL serum IgG (Figure 2B). When all intranasal immunizations are compared, mice immunized intranasally with CpG-IutA produced the greatest median concentration of antigen-specific antibody of all intranasal immunizations (44.9 µg/mL), although the response was highly variable between individual mice (Figure 2C) and did not correlate with a reduction in bladder bacterial burden (Supplemental Figure 3D).

Although intramuscular and subcutaneous immunization were less effective at reducing the bacterial burden in the urine, bladder and kidneys of challenged mice, these routes were able to generate an antigen-specific IgG response in the serum (Figure 3, Supplemental Figure 3). Intramuscular immunizations with MPLA produced the highest median concentration of antigen-specific antibody for all vaccine trials (median Hma 54.1 µg/mL, IreA 96.0 µg/mL) (Figure 3D), with vaccine combinations that used alum as adjuvant producing similar responses (Figure 3C). Immunizations performed with dmLT as adjuvant had a very weak IgG response when

281 administered intramuscularly (median Hma 5.7 µg/mL, IreA 8.3 µg/mL) (Figure 3A).
282 Subcutaneous immunizations produced a similar antigen-specific IgG response independent of
283 adjuvant, mean concentrations in pooled mouse sera ranging from 37.8 µg/mL to 97.3 µg/mL
284 (Supplemental Figure 3). Most notably, for both the intramuscular and subcutaneous routes,
285 immunization of mice with IreA increased the concentration of antigen-specific antibody above
286 similar vaccine formulations where Hma was used as antigen (Figure 3, Supplemental Figure 3),
287 indicating that IreA may be more antigenic despite having a highly similar tertiary structure.

Discussion

Currently, there is no licensed vaccine in the U.S to prevent UTI by *E. coli*. In this current era of increasing antibiotic resistance, developing preventive measures against urinary tract pathogens, especially UPEC, is critical. Towards that goal, here we have systematically tested four antigens, previously validated for their protective efficacy against colonization of the bladder and kidneys during experimental murine UTI (27, 28) in combination with each of five adjuvants, compatible with eventual clinical trial design, delivered by each of three routes. Intranasal immunization was previously protective in mice, while intramuscular or subcutaneous routes are more commonly used in humans. Intranasal administration of dmLT formulated with Hma or IutA significantly reduced bladder CFU and significantly increased the number of mice without detectable CFU. Immunization with dmLT which, like cholera toxin, is an A_1B_5 toxin, consistently generated a robust IgG antibody response against the administered antigen. Optimization of the route, adjuvant and antigen is an important step in development of a UTI vaccine for human clinical trials.

Induction of mucosal immune responses is most efficiently stimulated by delivery of a vaccine at a mucosal surface (61) and may be due to the common mucosal immune system that links tissues of the lung, gastrointestinal tract, urogenital tract, and nasopharynx (62). In mice, monkeys, and humans, vaccines administered intranasally generate mucosal immune responses in the female genital tract (63-66). This is attributed to the expression of redundant B and T cell homing receptors (CCR10, CCL28 and $\alpha_4\beta_7$-integrin) throughout mucosal surfaces, allowing circulating activated B and T cells to be attracted to multiple mucosal sites (67). Here we have shown that intranasal immunization is more effective at reducing bacterial CFU in the bladder of mice. Intramuscular and subcutaneous routes of vaccination may be less effective because

homing receptors are not expressed on B cells that are activated in peripheral lymph nodes (68). However, it has been shown that subcutaneous immunization performed with two weeks between boosts can significantly reduce kidney colonization and generate serum IgG in Balb/c mice when formulated with MPLA-FimH/MrpH (69), alum-FyuA (18) and adjuvant-IroN (17).

When administered intranasally, the adjuvant dmLT significantly reduced colonization of UPEC in the bladder during UTI. Other adjuvants were unable to produce equivalent results, independent of administration route. The success of dmLT recapitulates previous findings in experiments testing antigenicity of outer membrane iron receptors that utilized cholera toxin conjugated to antigens (27, 28). This is likely related to the similarity between the two toxins. Both are A_1B_5 toxins whose B subunits bind to ganglioside GM1 and facilitate endocytosis of the A subunit into the cytosol (70-72). In addition, we found that dmLT alone reduced colonization in the kidneys, consistent with other vaccines where dmLT was used as adjuvant (52). The mechanism of immune modulation for dmLT has been demonstrated to be a strong IL-17 response and activation of Th17 cells (52, 73), which may contribute to its success given the critical role of Th17 cells and IL-17 signaling during the host response to bladder infection (4, 28, 74).

The four antigens used to determine the optimal route and adjuvant for a urinary tract vaccine have similar cellular functions. These proteins display similar β-barrel structures, are located in the Gram-negative bacterial outer membrane, and mediate the uptake of siderophores (FyuA, IutA, and IreA) or heme molecules (Hma) from the extracellular environment. However, despite these similarities, there are marked differences in the host response when individually formulated with a single adjuvant. IutA was the only antigen to significantly reduce bacterial colonization in all sites of infection assessed. In addition, intranasal immunization with IutA

334 produced the most robust serum IgG response independent of adjuvant. This could be due to the
335 high percentage of its amino acid sequence likely to represent MHC class II epitopes predicted
336 by BepiPred-2.0, when compared to Hma, IreA, and FyuA. Furthermore, IutA has been shown to
337 be highly expressed in the mouse model of ascending UTI, as assessed by RNA microarray, and
338 in women presenting with symptoms of cystitis (32, 75, 76). Indeed, RNAseq data from 14
339 different strains, stabilized immediately following urine collection, clearly demonstrated that
340 *iutA, hma, fyuA,* and *ireA*, are highly expressed *in vivo* in women with acute cystitis (77). Based
341 on its efficacy and antigenicity, IutA should be considered for inclusion in future UTI vaccines,
342 particularly those aimed at increasing the breadth of protection against UPEC strains and
343 overcoming the functional redundancy of iron receptors by incorporation of multiple antigens.
344 Traditional vaccine development has been focused on production of antibodies and
345 immunological memory. Our intranasally administered vaccine using the adjuvant dmLT
346 significantly reduces *E. coli* colonization of the murine bladder when formulated with Hma or
347 IutA; however, serum antigen-specific antibody levels did not correlate with CFU in the current
348 study using purified LPS-free proteins. These results could suggest that administration of
349 adjuvants via our tested routes did not elicit an effective antibody response, and that cellular
350 immunity may be involved in protection. To that point, we previously demonstrated that
351 intranasal immunization with bacterial siderophores (yersiniabactin and aerobactin), that bind to
352 our iron-receptor antigens, provides protection by an unknown non-antibody-mediated
353 mechanism (16). In addition, experiments vaccinating mice with cholera toxin conjugated to
354 outer-membrane iron receptors generated antigen-specific antibody universally, independent of
355 any observed reduction in CFU during murine ascending UTI (28). Together these data suggest
356 that a strong IgG antibody response is not indicative of protection against infection. Further

357 work is required to identify the immune factors contributing to protection, which will

358 accelerate the development of an effective human UTI vaccine.

Materials and Methods

Ethics statement.

Animal protocols. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan Medical School (PRO00009173), and in accordance with the Office of Laboratory Animal Welfare (OLAW), the United States Department of Agriculture (USDA), and the guidelines specified by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC, Intl.). Mice were anesthetized with a weight-appropriate dose (0.1 mL for a mouse weighing 20 g) of ketamine/xylazine (80-120 mg/kg ketamine and 5-10 mg/kg xylazine) by intraperitoneal injection. Mice were euthanized by inhalant anesthetic overdose followed by vital organ removal.

Bacterial strains and culture conditions. *E. coli* CFT073 was isolated from the blood and urine of a hospitalized patient with acute pyelonephritis and urosepsis (78) and *E. coli* 536 was isolated from a patient with pyelonephritis (79). Transurethral infections with CFT073 or 536 were performed in mice immunized with vaccine formulations containing Hma, IreA, IutA, or FyuA. *E. coli* CFT073 does not encode a functional FyuA protein, thus, strain 536 was used to challenge mice that were immunized with FyuA. Strains were cultured in lysogeny broth (LB: 10 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl) at 37°C with aeration until saturation or on LB agar at 37°C.

Antigen purification and concentration. Commercially produced purified antigens were supplied by GenScript as follows. DNA sequences of *hma*, *ireA*, *iutA* from *E. coli* CFT073 and

382 *fyuA* from *E. coli* 536 were codon-optimized and individually synthesized in-frame with a 6X
383 histidine affinity tag and subcloned into *E. coli* expression vector pET-30a. Recombinant
384 plasmids were transformed into *E. coli* BL21 star (DE3), cultured with shaking at 37°C in TB
385 medium containing kanamycin, induced with IPTG, and harvested by centrifugation at 8,000
386 rpm. Cell pellets were lysed by sonication. Following centrifugation at 13,000 rpm, the
387 precipitate was dissolved using 8M urea. Target protein was filter sterilized using a 0.22 µm
388 membrane, quantified by the Bradford protein assay, and protein purity was determined by SDS-
389 PAGE and Western Blot. Purified protein was obtained from inclusion bodies with purity
390 varying from 85-90%. Proteins were certified as being endotoxin-free with levels < 100 EU/mg.
391 Prior to immunization and subsequent boosts protein was concentrated with 10,000 NMWL
392 centrifugal filter units (EMD Millipore).
393
394 **Vaccine formulation and administration.** Formulation of vaccines by mixing antigen and
395 adjuvant was performed on the day of primary immunization and on the day of each subsequent
396 boost. For each trial, seven to eight-week old CBA/J mice are given a primary dose of adjuvant
397 alone, or adjuvant combined with 100 µg purified, LPS-free antigen via the specified route,
398 intramuscular (IM), subcutaneous (SQ), or intranasal (IN), on day 0. On days 7 and 14, mice are
399 boosted with adjuvant alone, or adjuvant combined with 25 µg antigen by the same route. On
400 day 21 blood is collected retro orbitally via capillary tube, and mice are transurethrally
401 inoculated with a UPEC strain expressing the antigen of interest. Forty-eight hours post
402 inoculation urine is collected via abdominal massage and mice are sacrificed. Bladder and
403 kidneys are harvested and bacterial burden per mL urine or per gram tissue determined. Amount
404 and concentration of adjuvant for each vaccine was based upon pre-determined values as noted

405 in product specification sheets and in published studies (80-83). A total of five adjuvants were
406 tested for their efficacy within the vaccine formulations: Aluminum hydroxide gel (alum)
407 (Alhydrogel adjuvant 2%, InvivoGen), Polyinosinic-polycytidylic acid (poly (I:C) HMW)
408 (InvivoGen), Monophosphoryl Lipid A from *Salmonella minnesota* R595 (MPLA-SM)
409 (InvivoGen), unmethylated CpG synthetic oligodeoxynucleotides ODN 2395, type C (CpG)
410 (InvivoGen), and double mutant heat-labile toxin 1LT(R192G/L211A) (dmLT) (provided by Dr.
411 John D Clements and Dr. Elizabeth Norton; Tulane University School of Medicine) (84). See
412 Supplemental Table 2 for dose of adjuvant administered for each immunization route. For each
413 vaccine trial, a control vaccine formulation was prepared containing adjuvant alone and
414 phosphate buffered saline (PBS) with 1mM ethylenediamine tetraacetic acid (EDTA). Prepared
415 antigen formulations were administered to seven to eight-week old female CBA/J mice IN (20
416 μL/mouse, 10 μL/nare), IM (50 μL/mouse) or SQ (70 μL/mouse).

417

418 **Murine model of ascending UTI.** Female CBA/J mice were transurethrally challenged as
419 previously described (85). Briefly, bacterial pellets were harvested with centrifugation (3000 x *g*,
420 30 min, 4°C) and resuspended in phosphate-buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.44
421 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4) to a final dose of 2 x 10^9 CFU/mL. Actual inocula for
422 each experimental trial are compared in Supplemental Figure 1. Each mouse was anesthetized
423 and transurethrally infected with 50 μL of bacterial suspension using a Harvard Apparatus with a
424 flow rate of 100 μL/min. Forty-eight hours post inoculation blood and urine were collected,
425 mice were euthanized and bladder and kidneys were harvested. Urine and organ homogenates
426 were diluted, plated on LB agar using an Autoplate 4000 spiral plater (Spiral Biotech) and

enumerated using a QCount automated plate counter (Spiral Biotech) to determine the CFU/mL urine or CFU/g tissue.

Antibody quantification by ELISA. Quantification of antigen-specific antibody concentrations via indirect enzyme-linked immunosorbent assay (ELISA) was performed as previously described (27). Briefly, 5 μg/mL purified protein diluted in bicarbonate/carbonate buffer (3.03 g/L Na_2CO_3, 6.0 g/L $NaHCO_3$) was coated in each well and incubated at 4°C overnight. Plates were washed with PBST (PBS containing 0.05% Tween 20) using an ELx405 microplate washer (Bio-Teck Instruments, Inc.) and blocked with SuperBlock (Pierce). Following a second wash in PBST, 50 μL of sera diluted in SuperBlock or undiluted urine were added to wells and incubated for 1-2 h at room temperature. Plates were again washed with PBST and coated with 50 μL 1:10,000 diluted secondary antibody goat anti-mouse IgG HRP conjugated (1030-05, SouthernBiotech) and incubated 1 hr at room temperature. After a final wash in PBST, 50 μL 1-Step Ultra TMB (3,3',5,5'-tetramethylbenzidine) (Thermo Scientific) was added to each well and incubated at room temperature until sufficient color had developed. To stop the reaction, 50 μL 2M sulfuric acid was added to each well and the absorbance at 450 nm was read with a μQuant plate reader (Bio-Tek Instruments, Inc.). Antibody concentration was determined by comparing absorbance values to known concentrations of mouse IgG (0107-01, SouthernBiotech) bound to the plate with goat anti-mouse Ig (1010-01, SouthernBiotech). Serum assays were performed in duplicate for each mouse.

448 **Statistical analysis.** All graphic images and statistics were generated with Prism version 7
449 (GraphPad Software, Inc.). Significant differences in colonization levels and number of mice
450 without detectable CFU were determined by a two tailed Mann-Whitney test, and Fisher's exact
451 test, respectively. Correlations between antibody concentrations and bacterial burden were
452 determined by Pearson correlation coefficient.

453 ## Funding Information

454 This work was supported by the Public Health Service grant AI116791 from the National
455 Institutes of Health to HLT Mobley. The content may not represent the official views of the
456 National Institutes of Health.

457

458 ## Acknowledgements

459 We would like to thank all members of the Mobley laboratory for their insightful
460 comments and helpful critiques. We would also like to acknowledge Dr. John D. Clements and
461 Dr. Elizabeth Norton for the gift of dmLT.

Literature Cited

1. Dielubanza EJ, Schaeffer AJ. 2011. Urinary Tract Infections in Women. Medical Clinics of North America 95:27-41.
2. Sivick KE, Mobley HLT. 2010. Waging war against uropathogenic *Escherichia coli*: winning back the urinary tract. Infection and immunity 78:568-585.
3. O'Brien VP, Hannan TJ, Nielsen HV, Hultgren SJ. 2016. Drug and Vaccine Development for the Treatment and Prevention of Urinary Tract Infections. Microbiology spectrum 4:10.1128/microbiolspec.UTI-0013-2012.
4. Ingersoll MA, Albert ML. 2013. From infection to immunotherapy: host immune responses to bacteria at the bladder mucosa. Mucosal Immunology 6:1041.
5. Foxman B, Brown P. 2003. Epidemiology of urinary tract infections: Transmission and risk factors, incidence, and costs. Infectious Disease Clinics of North America 17:227-241.
6. Svanborg-Eden C, Svennerholm AM. 1978. Secretory immunoglobulin A and G antibodies prevent adhesion of *Escherichia coli* to human urinary tract epithelial cells. Infect Immun 22:790-7.
7. Riedasch G, Heck P, Rauterberg E, Ritz E. 1983. Does low urinary sIgA predispose to urinary tract infection? Kidney Int 23:759-63.
8. Modlin JF. 2004. Poliomyelitis in the United States: the final chapter? Jama 292:1749-51.
9. Kapikian AZ, Hoshino Y, Chanock RM, Perez-Schael I. 1996. Efficacy of a quadrivalent rhesus rotavirus-based human rotavirus vaccine aimed at preventing severe rotavirus diarrhea in infants and young children. J Infect Dis 174 Suppl 1:S65-72.
10. Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, Piedra P, Bernstein DI, Hayden FG, Kotloff K, Zangwill K, Iacuzio D, Wolff M. 1998. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. N Engl J Med 338:1405-12.
11. Levine MM. 2000. Immunization against bacterial diseases of the intestine. J Pediatr Gastroenterol Nutr 31:336-55.
12. Uehling DT, Wolf L. 1969. Enhancement of the bladder defense mechanism by immunization. Investigative urology 6:520-526.
13. Langermann S, Möllby R, Burlein JE, Palaszynski SR, Gale Auguste C, DeFusco A, Strouse R, Schenerman MA, Hultgren SJ, Pinkner JS, Winberg J, Guldevall L, Söderhäll M, Ishikawa K, Normark S, Koenig S. 2000. Vaccination with FimH Adhesin Protects Cynomolgus Monkeys from Colonization and Infection by Uropathogenic *Escherichia coli*. The Journal of Infectious Diseases 181:774-778.
14. Roberts JA, Kaack MB, Baskin G, Chapman MR, Hunstad DA, Pinkner JS, Hultgren SJ. 2004. Antibody Responses and Protection From Pyelonephritis Following Vaccination With Purified *Escherichia coli* PapDG Protein. Journal of Urology 171:1682-1685.
15. O'Hanley P, Lalonde G, Ji G. 1991. Alpha-hemolysin contributes to the pathogenicity of piliated digalactoside-binding *Escherichia coli* in the kidney: efficacy of an alpha-hemolysin vaccine in preventing renal injury in the BALB/c mouse model of pyelonephritis. Infection and Immunity 59:1153-1161.
16. Mike LA, Smith SN, Sumner CA, Eaton KA, Mobley HLT. 2016. Siderophore vaccine conjugates protect against uropathogenic *Escherichia coli* urinary tract infection.

Proceedings of the National Academy of Sciences of the United States of America 113:13468-13473.

17. Russo TA, McFadden CD, Carlino-MacDonald UB, Beanan JM, Olson R, Wilding GE. 2003. The Siderophore Receptor IroN of Extraintestinal Pathogenic *Escherichia coli* Is a Potential Vaccine Candidate. Infection and Immunity 71:7164-7169.
18. Habibi M, Asadi Karam MR, Bouzari S. 2017. Evaluation of prevalence, immunogenicity and efficacy of FyuA iron receptor in uropathogenic *Escherichia coli* isolates as a vaccine target against urinary tract infection. Microbial Pathogenesis 110:477-483.
19. Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HLT. 2009. Mucosal Immunization with Iron Receptor Antigens Protects against Urinary Tract Infection. PLOS Pathogens 5:e1000586.
20. Brumbaugh AR, Smith SN, Mobley HLT. 2013. Immunization with the yersiniabactin receptor, FyuA, protects against pyelonephritis in a murine model of urinary tract infection. Infection and immunity 81:3309-3316.
21. Cruz F, Dambros M, Naber KG, Bauer HW, Cozma G. 2009. Recurrent Urinary Tract Infections: Uro-Vaxom®, a New Alternative. European Urology Supplements 8:762-768.
22. Uehling DT, Hopkins WJ, Balish E, Xing Y, Heisey DM. 1997. Vaginal Mucosal Immunization for Recurrent Urinary Tract Infection: Phase II Clinical Trial. The Journal of Urology 157:2049-2052.
23. Huttner A, Hatz C, van den Dobbelsteen G, Abbanat D, Hornacek A, Frölich R, Dreyer AM, Martin P, Davies T, Fae K, van den Nieuwenhof I, Thoelen S, de Vallière S, Kuhn A, Bernasconi E, Viereck V, Kavvadias T, Kling K, Ryu G, Hülder T, Gröger S, Scheiner D, Alaimo C, Harbarth S, Poolman J, Fonck VG. 2017. Safety, immunogenicity, and preliminary clinical efficacy of a vaccine against extraintestinal pathogenic *Escherichia coli* in women with a history of recurrent urinary tract infection: a randomised, single-blind, placebo-controlled phase 1b trial. The Lancet Infectious Diseases 17:528-537.
24. Aziminia N, Hadjipavlou M, Philippou Y, Pandian SS, Malde S, Hammadeh MY. 2019. Vaccines for the prevention of recurrent urinary tract infections: a systematic review. BJU International 123:753-768.
25. Tammen H. 1990. Immunobiotherapy with Uro-Vaxom in Recurrent Urinary Tract Infection. British Journal of Urology 65:6-9.
26. Mobley LH, Alteri JC. 2016. Development of a Vaccine against *Escherichia coli* Urinary Tract Infections. Pathogens 5.
27. Brumbaugh AR, Smith SN, Mobley HL. 2013. Immunization with the yersiniabactin receptor, FyuA, protects against pyelonephritis in a murine model of urinary tract infection. Infect Immun 81:3309-16.
28. Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HL. 2009. Mucosal immunization with iron receptor antigens protects against urinary tract infection. PLoS Pathogens 5:e1000586.
29. Walters MS, Mobley HL. 2009. Identification of uropathogenic *Escherichia coli* surface proteins by shotgun proteomics. J Microbiol Methods 78:131-5.
30. Alteri CJ, Mobley HL. 2007. Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. Infect Immun 75:2679-88.
31. Snyder JA, Haugen BJ, Buckles EL, Lockatell CV, Johnson DE, Donnenberg MS, Welch RA, Mobley HL. 2004. Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. Infect Immun 72:6373-81.

32. Hagan EC, Lloyd AL, Rasko DA, Faerber GJ, Mobley HL. 2010. *Escherichia coli* global gene expression in urine from women with urinary tract infection. PLoS Pathog 6:e1001187.
33. Subashchandrabose S, Hazen TH, Brumbaugh AR, Himpsl SD, Smith SN, Ernst RD, Rasko DA, Mobley HL. 2014. Host-specific induction of *Escherichia coli* fitness genes during human urinary tract infection. Proc Natl Acad Sci U S A 111:18327-32.
34. Hagan EC, Mobley HL. 2007. Uropathogenic *Escherichia coli* outer membrane antigens expressed during urinary tract infection. Infect Immun 75:3941-9.
35. Spurbeck RR, Dinh PC, Jr., Walk ST, Stapleton AE, Hooton TM, Nolan LK, Kim KS, Johnson JR, Mobley HL. 2012. *Escherichia coli* isolates that carry *vat*, *fyuA*, *chuA*, and *yfcV* efficiently colonize the urinary tract. Infect Immun 80:4115-22.
36. Lloyd AL, Rasko DA, Mobley HL. 2007. Defining genomic islands and uropathogen-specific genes in uropathogenic *Escherichia coli*. J Bacteriol 189:3532-46.
37. Torres AG, Redford P, Welch RA, Payne SM. 2001. TonB-dependent systems of uropathogenic *Escherichia coli*: aerobactin and heme transport and TonB are required for virulence in the mouse. Infection and immunity 69:6179-6185.
38. Russo TA, Carlino UB, Johnson JR. 2001. Identification of a New Iron-Regulated Virulence Gene, *ireA*, in an Extraintestinal Pathogenic Isolate of *Escherichia coli*. Infection and immunity 69:6209-6216.
39. Hagan EC, Mobley HL. 2009. Haem acquisition is facilitated by a novel receptor Hma and required by uropathogenic *Escherichia coli* for kidney infection. Molecular microbiology 71:79-91.
40. Hancock V, Ferrieres L, Klemm P. 2008. The ferric yersiniabactin uptake receptor FyuA is required for efficient biofilm formation by urinary tract infectious *Escherichia coli* in human urine. Microbiology 154:167-175.
41. Levine MM, Kaper JB, Black RE, Clements ML. 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiological reviews 47:510-550.
42. R. E. 1997. Adjuvants for the future, p 173-192. *In* Levine MM WG, Kaper JB (ed), New Generation Vaccines. Marcel Dekker, Inc., New York.
43. Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. 2005. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. Vaccine 23:1588-1595.
44. Li H, Willingham SB, Ting JPY, Re F. 2008. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. Journal of immunology (Baltimore, Md : 1950) 181:17-21.
45. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science (New York, NY) 316:1628-1632.
46. Fransen F, Boog CJ, van Putten JP, van der Ley P. 2007. Agonists of Toll-like receptors 3, 4, 7, and 9 are candidates for use as adjuvants in an outer membrane vaccine against *Neisseria meningitidis* serogroup B. Infection and immunity 75:5939-5946.
47. Rhee EG, Kelley RP, Agarwal I, Lynch DM, La Porte A, Simmons NL, Clark SL, Barouch DH. 2010. TLR4 ligands augment antigen-specific CD8+ T lymphocyte responses elicited by a viral vaccine vector. Journal of virology 84:10413-10419.

48. Klinman DM, Barnhart KM, Conover J. 1999. CpG motifs as immune adjuvants. Vaccine 17:19-25.
49. Krieg AM, Davis HL. 2001. Enhancing vaccines with immune stimulatory CpG DNA. Current opinion in molecular therapeutics 3:15-24.
50. Sun S, Kishimoto H, Sprent J. 1998. DNA as an adjuvant: capacity of insect DNA and synthetic oligodeoxynucleotides to augment T cell responses to specific antigen. The Journal of experimental medicine 187:1145-1150.
51. Coffman RL, Sher A, Seder RA. 2010. Vaccine adjuvants: putting innate immunity to work. Immunity 33:492-503.
52. Clements JD, Norton EB. 2018. The Mucosal Vaccine Adjuvant LT(R192G/L211A) or dmLT. mSphere 3:e00215-18.
53. Hogenesch H. 2013. Mechanism of immunopotentiation and safety of aluminum adjuvants. Frontiers in immunology 3:406-406.
54. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. 2007. The Vaccine Adjuvant Monophosphoryl Lipid A as a TRIF-Biased Agonist of TLR4. Science 316:1628-1632.
55. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, Kielland A, Vosters O, Vanderheyde N, Schiavetti F, Larocque D, Van Mechelen M, Garçon N. 2009. AS04, an Aluminum Salt- and TLR4 Agonist-Based Adjuvant System, Induces a Transient Localized Innate Immune Response Leading to Enhanced Adaptive Immunity. The Journal of Immunology 183:6186-6197.
56. Storni T, Ruedl C, Schwarz K, Schwendener RA, Renner WA, Bachmann MF. 2004. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. J Immunol 172:1777-85.
57. Chaput N, Schartz NE, Andre F, Taieb J, Novault S, Bonnaventure P, Aubert N, Bernard J, Lemonnier F, Merad M, Adema G, Adams M, Ferrantini M, Carpentier AF, Escudier B, Tursz T, Angevin E, Zitvogel L. 2004. Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. J Immunol 172:2137-46.
58. Wettstein PJ, Borson ND, Park JG, McNallan KT, Reed AM. 2005. Cysteine-tailed class I-binding peptides bind to CpG adjuvant and enhance primary CTL responses. J Immunol 175:3681-9.
59. Kim YC, Jarrahian C, Zehrung D, Mitragotri S, Prausnitz MR. 2012. Delivery systems for intradermal vaccination. Current topics in microbiology and immunology 351:77-112.
60. Coleman BL, McGeer AJ, Halperin SA, Langley JM, Shamout Y, Taddio A, Shah V, McNeil SA. 2012. A randomized control trial comparing immunogenicity, safety, and preference for self- versus nurse-administered intradermal influenza vaccine. Vaccine 30:6287-6293.
61. Neutra MR, Kozlowski PA. 2006. Mucosal vaccines: the promise and the challenge. Nature Reviews Immunology 6:148-158.
62. Gebril A, Alsaadi M, Acevedo R, Mullen AB, Ferro VA. 2012. Optimizing efficacy of mucosal vaccines. Expert review of vaccines 11:1139-1155.
63. Kozlowski PA, Williams SB, Lynch RM, Flanigan TP, Patterson RR, Cu-Uvin S, Neutra MR. 2002. Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. Journal of immunology (Baltimore, Md : 1950) 169:566-574.

64. Staats HF, Montgomery SP, Palker TJ. 1997. Intranasal immunization is superior to vaginal, gastric, or rectal immunization for the induction of systemic and mucosal anti-HIV antibody responses. AIDS research and human retroviruses 13:945-952.
65. Imaoka K, Miller CJ, Kubota M, McChesney MB, Lohman B, Yamamoto M, Fujihashi K, Someya K, Honda M, McGhee JR, Kiyono H. 1998. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. Journal of immunology (Baltimore, Md : 1950) 161:5952-5958.
66. Rudin A, Riise GC, Holmgren J. 1999. Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. Infection and immunity 67:2884-2890.
67. Mestecky J. 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. Journal of clinical immunology 7:265-276.
68. Kunkel EJ, Butcher EC. 2003. Plasma-cell homing. Nature Reviews Immunology 3:822-829.
69. Habibi M, Asadi Karam MR, Bouzari S. 2015. Evaluation of the effect of MPL and delivery route on immunogenicity and protectivity of different formulations of FimH and MrpH from uropathogenic *Escherichia coli* and *Proteus mirabilis* in a UTI mouse model. International Immunopharmacology 28:70-78.
70. Lencer WI, Saslowsky D. 2005. Raft trafficking of AB5 subunit bacterial toxins. Biochimica et biophysica acta 1746:314-321.
71. Wang H, Paton JC, Herdman BP, Rogers TJ, Beddoe T, Paton AW. 2013. The B subunit of an AB5 toxin produced by *Salmonella enterica serovar Typhi* up-regulates chemokines, cytokines, and adhesion molecules in human macrophage, colonic epithelial, and brain microvascular endothelial cell lines. Infection and immunity 81:673-683.
72. Beddoe T, Paton AW, Le Nours J, Rossjohn J, Paton JC. 2010. Structure, biological functions and applications of the AB5 toxins. Trends in biochemical sciences 35:411-418.
73. Norton EB, Lawson LB, Mahdi Z, Freytag LC, Clements JD. 2012. The A Subunit of *Escherichia coli* Heat-Labile Enterotoxin Functions as a Mucosal Adjuvant and Promotes IgG2a, IgA, and Th17 Responses to Vaccine Antigens. Infection and Immunity 80:2426-2435.
74. Sivick KE, Schaller MA, Smith SN, Mobley HL. 2010. The innate immune response to uropathogenic *Escherichia coli* involves IL-17A in a murine model of urinary tract infection. The journal of immunology 184:2065-2075.
75. Snyder JA, Haugen BJ, Buckles EL, Lockatell CV, Johnson DE, Donnenberg MS, Welch RA, Mobley HLT. 2004. Transcriptome of uropathogenic *Escherichia coli* during urinary tract infection. Infection and immunity 72:6373-6381.
76. Hagan EC, Lloyd AL, Rasko DA, Faerber GJ, Mobley HLT. 2010. *Escherichia coli* global gene expression in urine from women with urinary tract infection. PLoS pathogens 6:e1001187-e1001187.
77. Sintsova A, Frick-Cheng AE, Smith S, Pirani A, Subashchandrabose S, Snitkin ES, Mobley H. 2019. Genetically diverse uropathogenic *Escherichia coli* adopt a common transcriptional program in patients with UTIs. eLife 8:e49748.

78. Mobley HL, Green DM, Trifillis AL, Johnson DE, Chippendale GR, Lockatell CV, Jones BD, Warren JW. 1990. Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infection and immunity 58:1281-1289.
79. Hacker J, Knapp S, Goebel W. 1983. Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of an *Escherichia coli* O6 strain. Journal of bacteriology 154:1145-1152.
80. Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S-I, Takahashi H, Sawa H, Chiba J, Kurata T, Sata T, Hasegawa H. 2005. Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. Journal of virology 79:2910-2919.
81. Marshall JD, Fearon KL, Higgins D, Hessel EM, Kanzler H, Abbate C, Yee P, Gregorio J, Cruz TD, Lizcano JO, Zolotorev A, McClure HM, Brasky KM, Murthy KK, Coffman RL, Nest GV. 2005. Superior Activity of the Type C Class of ISS In Vitro and In Vivo Across Multiple Species. DNA and Cell Biology 24:63-72.
82. Romero CD, Varma TK, Hobbs JB, Reyes A, Driver B, Sherwood ER. 2011. The Toll-like receptor 4 agonist monophosphoryl lipid a augments innate host resistance to systemic bacterial infection. Infection and immunity 79:3576-3587.
83. Lindblad EB, Schønberg NE. 2010. Aluminum Adjuvants: Preparation, Application, Dosage, and Formulation with Antigen, p 41-58. *In* Davies G (ed), Vaccine Adjuvants: Methods and Protocols doi:10.1007/978-1-60761-585-9_4. Humana Press, Totowa, NJ.
84. Norton EB, Lawson LB, Freytag LC, Clements JD. 2011. Characterization of a mutant *Escherichia coli* heat-labile toxin, LT(R192G/L211A), as a safe and effective oral adjuvant. Clinical and vaccine immunology : CVI 18:546-551.
85. Johnson DE, Lockatell CV, Hall-Craigs M, Mobley HL, Warren JW. 1987. Uropathogenicity in rats and mice of *Providencia stuartii* from long-term catheterized patients. J Urol 138:632-5.

Figure Legends

Figure 1. Comparison of adjuvants administered intranasally and formulated with the antigens Hma, IutA, FyuA, or IreA. Seven to eight-week old female CBA/J mice were immunized intranasally according to our immunization schedule with adjuvant alone (Ctl) or adjuvant formulated with 100 µg LPS free antigen, either Hma (A, E, I), IutA (B, F, J), FyuA (C, G, K), or IreA (D, H, L). Adjuvants tested included unmethylated CpG synthetic oligodeoxynucleotides (CpG), polyinosinic:polycytodylic acid (polyIC), or detoxified *E. coli* enterotoxin (dmLT). One week after the final boost, mice were challenged with 10^8 colony forming units (CFU) of *E. coli* strain CFT073 (Hma, IreA, IutA) or 536 (FyuA) via transurethral inoculation. Forty-eight hours post inoculation, urine was collected, mice were sacrificed, bladder and kidneys homogenized, and aliquots plated on LB agar for enumeration of bacterial burden. Bars indicate the median CFU in the urine (A - D), bladder (E - H), and kidneys (I – L). Symbols represent individual mice. N = 5 - 30. Dashed line represents the limit of detection. *P* values were determined using a two-tailed Mann-Whitney test.

Figure 2. Intranasal vaccination with outer membrane iron receptors generates a robust antigen-specific serum IgG response. Antigen-specific IgG concentrations quantified by indirect ELISA in serum collected from female CBA/J mice one week after final boost. Mice were intranasally immunized with adjuvant alone (Ctl) or adjuvant formulated with 100 µg of purified, LPS free antigen (Hma, IreA, FyuA, IutA). Adjuvants utilized were (A) detoxified *E. coli* enterotoxin, dmLT, (B) polyinosinic:polycytodylic acid, polyIC, and (C) unmethylated CpG

synthetic oligodeoxynucleotides, CpG. Each bar represents the median and each symbol represents an individual mouse. N = 5 – 20. *P* values were determined using a two-tailed Mann-Whitney test. *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Figure 3. Intramuscular vaccination with outer membrane iron receptors generates a robust antigen-specific serum IgG response when alum or MPLA are used as adjuvants. Antigen-specific IgG concentrations quantified by indirect ELISA in serum collected from female CBA/J mice one week after final boost. Mice were intramuscularly immunized with adjuvant alone (Ctl) or adjuvant formulated with 100 µg of purified, LPS free antigen (Hma, IreA, FyuA, IutA). Adjuvants utilized were (A) detoxified *E. coli* enterotoxin, dmLT, (B) polyinosinic:polycytodylic acid, polyIC, (C) alum, and (D) monophosphoryl lipid A, MPLA. Note the change in scale in panel D. Each bar represents the median and each symbol represents an individual mouse. N = 10 – 20. *P* values were determined using a two-tailed Mann-Whitney test. *** $P \leq 0.001$, ** $P \leq 0.01$.

Supplemental Figure Legends

Supplemental Figure 1. Inocula are consistent across experimental trials and strains. Inoculating doses of UPEC in CFU/mL administered transurethrally to female CBA/J mice one week following final boost. Mice were inoculated with strain CFT073 following immunization with Hma, IreA or IutA, or inoculated with strain 536 following immunization with FyuA. The intended dose was 2 x 10^9 CFU/mL. Whiskers indicate maximum and minimum, box indicates

759 25th and 75th percentiles, bar indicates the median. N = 6 – 26. No statistical difference was
760 found via two-tailed Mann-Whitney test.

761

762 **Supplemental Figure 2. Immunizing with CpG or polyIC in the absence of antigen does not**
763 **affect colonization of the urinary tract by UPEC.** Seven to eight-week old female CBA/J mice
764 were immunized intranasally according to our immunization schedule with the adjuvants:
765 unmethylated CpG synthetic oligodeoxynucleotides (CpG), detoxified *E. coli* enterotoxin
766 (dmLT), or polyinosinic:polycytodylic acid (polyIC). One week after the final boost, mice were
767 transurethrally challenged with 10^8 CFU of CFT073. Forty-eight hours post inoculation, urine
768 was collected, mice were sacrificed, bladder and kidneys homogenized, and aliquots plated on
769 LB agar for enumeration of bacterial burden. Bars indicate the median CFU in the urine (A),
770 bladder (B), and kidneys (C). Symbols represent individual mice. N = 5-40. The limit of
771 detection is 100 CFU/mL urine or /g tissue. Dotted line represents colonization level observed in
772 unimmunized mice challenged with CFT073. *P* values shown where addition of adjuvant was
773 protective as determined using a two-tailed Mann-Whitney test.

774

775 **Supplemental Figure 3. Subcutaneous vaccination with outer membrane iron receptors**
776 **generates an antigen-specific serum IgG response.** Antigen-specific IgG concentrations
777 quantified by indirect ELISA in pooled serum collected from female CBA/J mice one week after
778 final boost. Mice were subcutaneously immunized with adjuvant alone (Ctl) or adjuvant
779 formulated with 100 µg of purified, LPS free antigen (Hma, IreA, FyuA, IutA). Adjuvants
780 utilized were: detoxified *E. coli* enterotoxin, (dmLT), polyinosinic:polycytodylic acid (polyIC),

781 alum, and monophosphoryl lipid A (MPLA). Each bar represents the mean from four technical
782 replicates of serum pooled from 10 mice from either one or two experimental trials, error bars
783 indicate standard deviation. *P* values were determined using a two-tailed Mann-Whitney test. **
784 $P \leq 0.01$, * $P \leq 0.05$.

785

786 **Supplemental Figure 4. Antigen-specific serum antibodies do not correlate with protection**
787 **against UTI.** The CFU/mL urine or /g tissue (y-axis) of all vaccination trials that significantly
788 reduced bacterial burden (see Figure 1 and 3) were correlated with antigen-specific serum IgG
789 concentration measured by ELISA (x-axis). Seven to eight-week old female CBA/J mice were
790 immunized intranasally according to our immunization schedule with detoxified *E. coli*
791 enterotoxin (dmLT) formulated with Hma (A), dmLT-IutA (B), polyinosinic:polycytodylic acid
792 formulated with IutA (C), or unmethylated CpG synthetic oligodeoxynucleotides formulated
793 with IutA (D). Each symbol represents a single mouse. Open symbols represent mice immunized
794 with adjuvant alone, closed symbols represent mice immunized with adjuvant formulated with
795 antigen. N = 5-17. No significant correlations were found using Pearsons correlation coefficient.

796

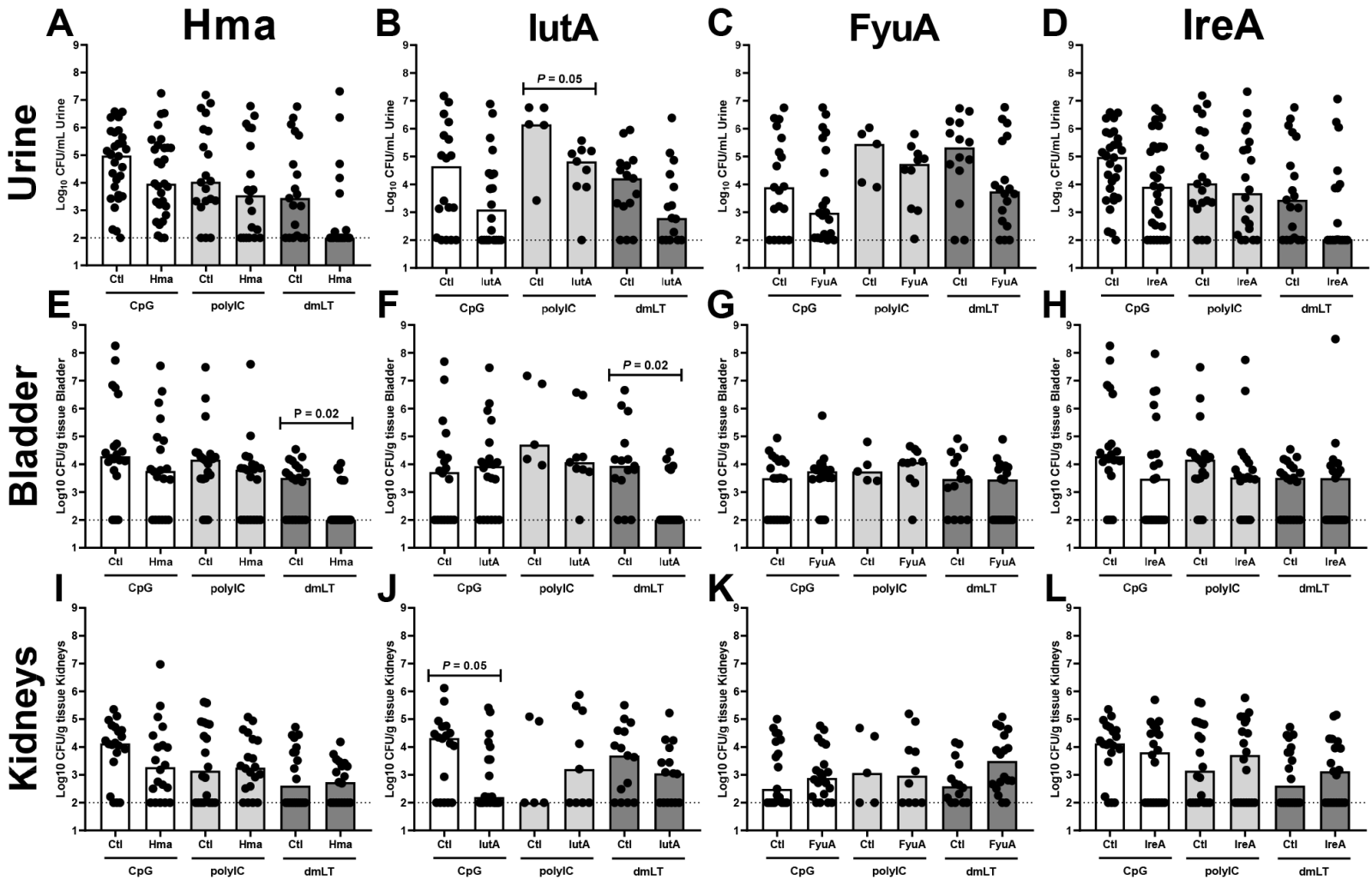
797 **Table 1. Median fold change in CFU in the urine, bladder and kidneys of immunized mice.**

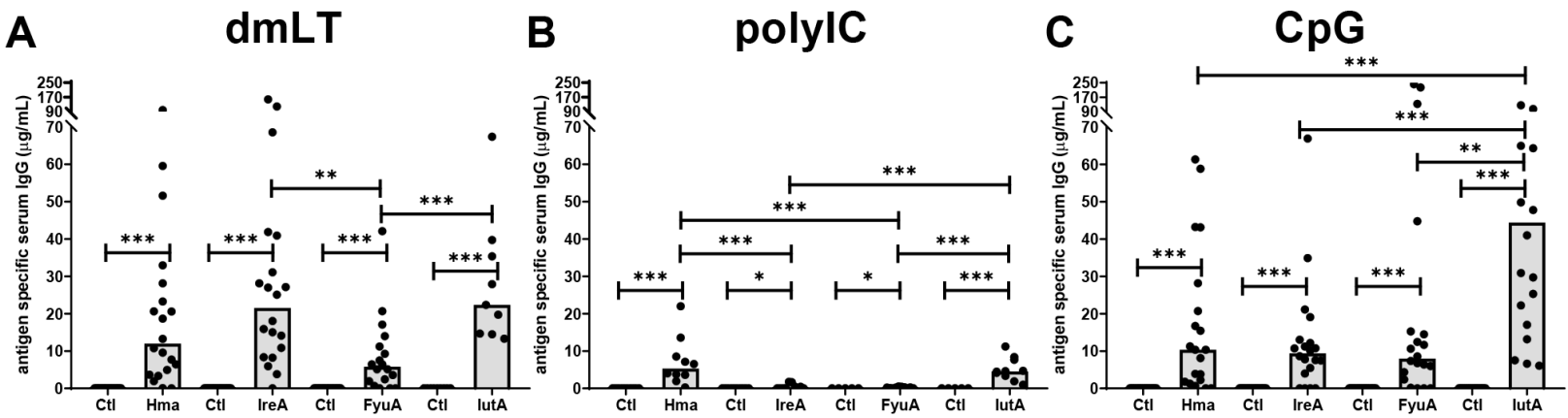
798

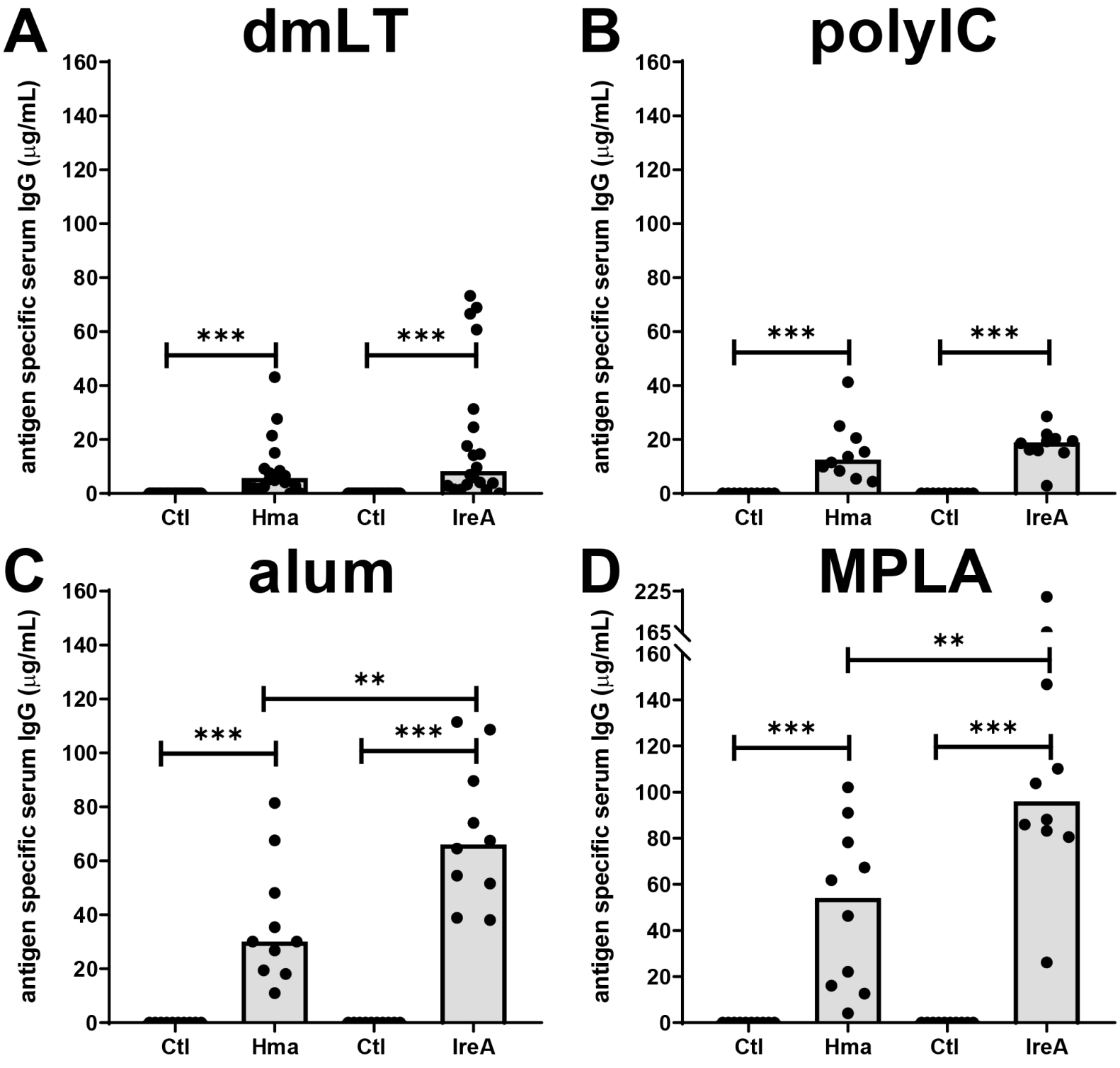
799 **Supplemental Table 1. Percent of immunized mice without detectable CFU following**
800 **transurethral challenge.**

801

802 **Supplemental Table 2. Doses for each adjuvant by route of administration.**







			Urine				Bladder				Kidneys			
Route	Adjuvant	Antigen	Median Adj Only[a]	Median Adj + Antigen[b]	Fold Change[c]	P value[d]	Median Adj Only	Median Adj + Antigen	Fold Change	P value	Median Adj Only	Median Adj + Antigen	Fold Change	P value
IM[e]	alum	Hma	1840	105300	0.02	**0.0257**	100	8670	0.01	0.2628	10940	6860	1.59	0.9861
IM	alum	IreA	1840	7285	0.25	0.0664	100	2950	0.03	0.9709	10940	10045	1.09	0.5761
IM	polyIC[f]	Hma	23100	734500	0.03	0.4973	6735	37350	0.18	**0.0522**	9650	3975	**2.43**	0.2834
IM	polyIC	IreA	23100	106000	0.22	0.6421	6735	13550	0.50	0.5326	9650	750	**12.87**	0.1808
IM	MPLA[g]	Hma	105350	562500	0.19	0.4813	100	4810	0.02	0.3285	3455	13620	0.25	0.3888
IM	MPLA	IreA	105350	17100	**6.16**	0.2466	100	1690	0.06	0.7799	3455	470	**7.35**	0.8994
IM	dmLT[h]	Hma	218500	101100	**2.16**	0.3440	28200	92650	0.30	0.4311	40400	6650	**6.08**	0.2400
IM	dmLT	IreA	218500	36500	**5.99**	0.1127	28200	14500	1.94	0.7275	40400	11000	**3.67**	0.7252
IN[i]	polyIC	Hma	10950	3500	**3.13**	0.2409	14600	6355	**2.30**	0.1009	1409	1810	0.78	>0.9999
IN	polyIC	IreA	10950	4845	**2.26**	0.4122	14600	3330	**4.38**	0.1457	1409	5125	0.27	0.7937
IN	polyIC	FyuA	284000	54100	**5.25**	0.3097	5500	11800	0.47	0.5941	1180	927	1.27	>0.9999
IN	polyIC	IutA	1440000	66700	**21.59**	**0.0599**	50900	11800	**4.31**	0.1469	640	1600	0.40	0.5804
IN	dmLT	Hma	2790	100	**27.90**	0.0693	3205	100	**32.05**	**0.0240**	407	542	0.75	0.6033
IN	dmLT	IreA	2790	105	**26.57**	0.3674	3205	3170	1.01	0.9020	407	1340	0.30	0.7087
IN	dmLT	FyuA	210450	5605	**37.55**	0.0825	2955	2835	1.04	0.4233	387	3159	0.12	**0.0476**
IN	dmLT	IutA	16400	619	**26.49**	0.1866	8610	100	**86.10**	**0.0181**	4950	1130	**4.38**	0.2536
IN	CpG[j]	Hma	97050	9285	**10.45**	0.4332	19350	5755	**3.36**	0.1321	13500	1885	**7.16**	0.1419
IN	CpG	IreA	97050	8215	**11.81**	0.4944	19350	3035	**6.38**	0.0608	13500	6490	**2.08**	0.2991
IN	CpG	FyuA	7910	961	**8.23**	0.6403	3130	5625	0.56	0.8550	312	773	0.40	0.8330
IN	CpG	IutA	44530	1240	**35.91**	0.1959	5255	8570	0.61	0.7791	21100	161	**131.06**	**0.0462**
SQ[k]	alum	Hma	2218	100	**22.18**	0.1246	3240	8205	0.39	0.7940	198	100	1.98	0.2391
SQ	alum	IreA	2218	3165	0.70	0.7828	3240	4655	0.70	0.9271	198	2315	0.09	0.3376
SQ	polyIC	Hma	100	1705	0.06	0.7168	1505	14650	0.10	**0.0052**	100	100	1.00	0.4241
SQ	polyIC	IreA	100	1710	0.06	0.5473	1505	4410	0.34	0.2559	100	9615	0.01	**0.0274**
SQ	dmLT	Hma	119000	8955	**13.29**	0.3154	2750	2555	1.08	0.9478	4390	12440	0.35	0.4422
SQ	dmLT	IreA	119000	213500	0.56	0.9048	2750	11360	0.24	0.7122	4390	4990	0.88	0.9675
SQ	MPLA	Hma	31900	232600	0.14	0.7988	5240	13800	0.38	0.5935	41800	5615	**7.44**	0.2818
SQ	MPLA	IreA	31900	202000	0.16	0.4002	5240	14700	0.36	0.0810	41800	54650	0.76	0.2309

Table 1. Median fold change in CFU in the urine, bladder and kidneys of immunized mice

[a]Median CFU when immunized with the adjuvant alone.
[b]Median CFU when immunized with the adjuvant formulated with antigen.
[c]Fold change in median CFU when immunized with the adjuvant alone compared to mice immunized with the adjuvant formulated with antigen. Fold change greater than 2 are shown in bold.
[d]*P* value as determined by two tailed Mann-Whitney exact test. Significant differences are shown in bold.

[e]Intramuscular [f]Polyinosinic:polycytodylic acid [g]Monophosphoryl lipid A [h]Detoxified *E. coli* enterotoxin [i]Intranasal
[j]Unmethylated CpG synthetic oligodeoxynucleotides [k]Subcutaneous