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(54) Title: NGAL AND URINARY TRACT INFECTION

(57) Abstract: In some embodiments, the present invention is directed to compositions and methods for the treatment and prevention of urinary tract infections (UTIs) and urosepsis. In some embodiments, the methods of the invention comprise administering to a subject in need thereof a therapeutically effective amount of an agent that stimulates genito-urinary tract epithelial cells to produce NGAL, and/or an NGAL protein or a functional derivative thereof, and optionally also administering to the subject an additional agent useful for treating or preventing UTI or urosepsis. In other embodiments, the present invention also provides methods of screening for agents that stimulate urinary tract epithelial cells to produce NGAL.



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NGAL AND URINARY TRACT INFECTION

[0001] This application claims the benefit of the filing date of U.S. Provisional Patent Application No. 61/332,477, filed May 7, 2010, and U.S. Provisional Patent Application No. 61/347,954, filed May 25, 2010, the contents of each of which are hereby incorporated by reference.

[0002] For the purposes only of the U.S. and other jurisdictions that permit incorporation by reference, all patents, patent applications and publications cited herein are hereby incorporated by reference in their entireties.

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BACKGROUND

[0004] Urinary tract infections or “UTIs” are one of the most prevalent and resource taxing diseases in the U.S., with 13.3% (12.8 million) of all women and 2.3% (2 million) of all men in the U.S. infected annually, resulting in an annual cost to the U.S. healthcare system of around \$3.5 billion. In 2000 there were an estimated 11.02 million visits (2.05 million men; 8.97 million women) to a physician’s office or hospital related to UTI. Uropathogenic *Escherichia coli* or “UPEC” bacteria are involved in 70-95% of all cases of UTI. Many of these UPEC bacteria rely on catecholate-siderophores as their primary iron uptake mechanism.

[0005] Neutrophil Gelatinase Associated Lipocalin (NGAL) is a small secreted protein with a molecular weight of about 22kD, and is a siderophore and iron binding protein. A siderophore is an organic molecule that binds to and chelates iron. Bacteria produce siderophores such as enterochelin. Mammals endogenously produce a siderophore called catechol. Enterochelin has an extremely high affinity for iron, and NGAL has a high affinity for the enterochelin-iron complex.

SUMMARY OF THE INVENTION

[0006] The present invention is based, in part, on certain discoveries which are described more fully in the Examples section of the present application. For example, the present

invention is based, in part, on the discovery that in response to infection of the urinary tract with enterochelin-dependent uropathogenic bacteria, epithelial cells of the genitourinary tract secrete NGAL protein, which has bacteriostatic activity and inhibits growth of bacteria. The present invention is also based, in part, on the elucidation of the biochemical pathways that result in the secretion of NGAL protein by epithelial cells of the genitourinary tract in response to a urinary tract infection.

[0007] In one embodiment, the present invention provides a method for treating or preventing infection of the urinary tract with a bacterium, such as an enterochelin-dependent uropathogenic bacterium, in a subject, or treating or preventing urosepsis resulting from such an infection, the method comprising administering to the subject a therapeutically effective amount of one or more agents selected from the group consisting of: (a) an agent that stimulates genito-urinary tract epithelial cells of the subject to secrete NGAL protein, (b) NGAL, and (c) a functional derivative thereof, or combinations of one or more thereof. In some embodiments, the bacterium is an enterochelin-dependent bacterium, such as an enterochelin-dependent uropathogenic *E. coli* or “UPEC” bacterium. In some embodiments the agent that stimulates genito-urinary tract epithelial cells to secrete NGAL protein stimulates the secretion of NGAL by epithelial cells of the kidney, such as epithelial cells of the collecting duct or epithelial cells of the thick ascending limb of Henlé in the kidney. In some embodiments, the agent that stimulates genito-urinary tract epithelial cells of the subject to secrete NGAL protein is an NFκB activator, an activator of a TLR-NFκB pathway (such as an activator of a TLR4-NFκB or TLR11-NFκB pathway), a NRF2 modulator, a HIF modulator, or a non-toxic derivative of either lipid A, lipopolysaccharide, or endotoxin. In some embodiments the agents are administered systemically. In other embodiments the agents are administered locally.

[0008] In another embodiment, the present invention provides a method for treating or preventing infection of the urinary tract with a bacterium, such as an enterochelin-dependent uropathogenic bacterium, in a subject, or treating or preventing urosepsis resulting from such an infection, the method comprising stimulating genito-urinary tract epithelial cells of the subject to secrete NGAL protein. In some embodiments, the bacterium is an enterochelin-dependent uropathogenic bacterium, such as an enterochelin-dependent uropathogenic *E. coli* (UPEC) bacterium. In some embodiments, the genito-urinary tract epithelial cells are kidney epithelial cells, such as epithelial cells of the collecting duct, or epithelial cells of the thick ascending limb of Henlé. In some embodiments the step of stimulating genito-urinary tract

epithelial cells to secrete NGAL protein comprises administering to the subject a therapeutically effective amount one or more agent selected from the group consisting of: (a) a non-toxic derivative of lipid A, (b) a non-toxic derivative of lipopolysaccharide, (c) a non-toxic derivative of endotoxin, (d) an activator of the TLR4-NFκB pathway, (e) an activator of the TLR11-NFκB pathway, (f) an NFκB activator, (g) a NRF2 modulator, and (h) a HIF modulator, or a combination of one or more thereof. In some embodiments such agents are administered systemically to the subject. In other embodiments such agents are administered locally to the genitourinary tract of the subject.

[0009] In other embodiments, the present invention provides pharmaceutical compositions for use in treating a urinary tract infection or urosepsis, the compositions comprising a therapeutically effective amount of an agent that stimulates genito-urinary tract epithelial cells to secrete NGAL protein, and optionally a therapeutically effective amount of NGAL, or a functional derivative thereof. In some embodiments the agent that stimulates genito-urinary tract epithelial cells to secrete NGAL protein is selected from the group consisting of: (a) a non-toxic derivative of lipid A, (b) a non-toxic derivative of lipopolysaccharide, (c) a non-toxic derivative of endotoxin, (d) an activator of the TLR4-NFκB pathway, (e) an activator of the TLR11-NFκB pathway, (f) an NFκB activator, (g) a NRF2 modulator, and (h) a HIF modulator.

[0010] In yet other embodiments, the present invention provides methods of screening for agents that stimulate epithelial cells of the urinary tract, such as kidney epithelia cells (including epithelial cells of the collecting ducts or of the thick ascending limb of Henlé), bladder epithelial cells, and urethral epithelial cells, to produce NGAL mRNA or protein. In some embodiments, such screening methods comprise providing a population of urinary tract epithelial cells, contacting the population of urinary tract epithelial cells with one or more test agents, and testing for production of NGAL mRNA or protein by the urinary tract epithelial cells, thereby identifying agents that stimulate production of NGAL mRNA or protein by the urinary tract epithelial cells. In some such embodiments, the urinary tract epithelial cells may be *in vivo*, for example in a mouse model. In other embodiments, the urinary tract epithelial cells may be cultured *in vitro*. Urinary tract epithelial cells that are cultured *in vitro* may be primary cultures, or may be derived from primary cultures, or may be cell lines, such as established urinary tract epithelial cell lines, including kidney epithelial cell lines, bladder epithelial cells lines, urethral epithelial cell lines, and the like. The test agents may be any suitable test agents, including, but not limited to, libraries of small molecule drugs, libraries

of proteinaceous or peptide drugs (including peptidomimetic drugs), libraries of antibodies, libraries of RNA molecules (including, but not limited to, antisense RNAs, siRNAs, shRNAs, and microRNAs, ribozymes), and the like. In addition to libraries of test agents, individual test agents, or smaller populations of test agents, may also be used. Any suitable means may be used to detect NGAL production by the urinary tract epithelial cells. In one embodiment, secreted NGAL protein is detected in cell supernatants. In another embodiment, NGAL protein within the epithelial cells is detected. NGAL protein may be detected using any suitable means. In one embodiment, NGAL protein is detected using an antibody to NGAL. The NGAL antibody may be labeled with a detectable moiety, or a secondary antibody that is labeled with a detectable moiety may be used. Suitable detectable moieties may include enzyme substrates (such as horseradish peroxidase, alkaline phosphatase, and the like), and fluorescent labels (such as green fluorescent protein, and the like). In one embodiment NGAL protein may be detected in an ELISA assay using an anti-NGAL antibody. In another embodiment NGAL mRNA is detected. NGAL mRNA may be detected using any suitable means, including, but not limited to, *in situ* hybridization, Northern blotting, PCR, QPCR, and the like. Any suitable probes or primers for detection of NGAL mRNA may be used.

[0011] These and other embodiments of the invention are further described in the following sections of the application, including the Detailed Description, Examples, Claims, and Drawings.

BRIEF DESCRIPTION OF THE FIGURES

[0012] **Figure 1:** The kidney is an exocrine organ which defends the urinary system from infection by secreting NGAL. NGAL has been shown to be a binding protein for gram-negative siderophores such as enterochelin. To establish whether uNGAL can inhibit the growth of a highly pathogenic uropathogenic *Escherichia coli* strain (CFT073) NGAL activity was investigated both *in vitro* and *in vivo*. (a) An *in vitro* culture of CFT073 UPEC in minimal media (M9), M9 containing iron (1mM FeCl₃), M9 and uNGAL (5μM), and M9 with uNGAL and high iron, showed that the presence of uNGAL can inhibit growth of the UPEC and that inhibition could be reversed by addition of high iron. Bacterial growth was assessed by measuring the optical density at indicated time points. Results from each treatment were compared with the results from Medium alone using unpaired T test. (b) To determine the *in vivo* role of uNGAL, sibling matched wild-type animals and Ngal globally deleted animals (EIIa-cre) were challenged with a transurethral (TU) injection of CFT073. CFT073 presence in the GU correlated to uNGAL as seen in the Western blot (WB). Ngal

deleted animals compared to wild-type (WT) took significantly longer to clear a CFT073 infection - 6 days compared to 3 days for a wild type.

[0013] Figure 2: Generation of the NgalloxP/loxP conditional knockout murine model. (a) A loxP site was inserted into intron 1 (light gray arrowhead), a neomycin cassette (light blue box) into intron 5 flanked by FRT (dark gray arrowhead) and loxP sites on the 5' and 3' end. Neomycin cassette was excised by breeding the heterozygous NgalloxPneo/+ with a Flippase Recombinase (FLP) mouse to make the Ngalloxp/+. Ngalloxp/+ was bred against C57BL/6 animals to remove the FLP gene. Ngalloxp/+ were subsequently bred against EIIa-Cre mice for site-specific recombination between the intron 1 loxP site and the intron 5 loxP site creating a NgalloxP/+, EIIa-Cre mouse. (b) Genotyping strategy of the Ngal loxP-cre system.

[0014] Figure 3: The distal nephron and the bladder epithelium responds to an uropathogenic infection by secreting uNGAL. (a, b) *In situ* hybridization demonstrated Ngal mRNA expression in the bladder epithelium (a) and in collecting ducts (CD) of the kidney (b) 24 hours after an acute UTI (10-20 μ l of 5×10^9 CFU/ml CFT073). Ngal mRNA was predominantly expressed in a few CD and was absent all other epithelial tubules and the thin limb of Henle. (c) In order to determine the contribution of uNGAL from the kidney and the bladder, QPCR was done 1 day post-TU and Ngal copy number was determined. Almost 3 log orders greater Ngal copies increase were observed in the kidney compared to the bladder.

[0015] Figure 4: Toll dependence of NGAL expression in Kidney. (a) LPS-insensitive mice, C3H/HeJ have significantly less uNGAL than wild type animals after i.p. challenge with LPS. To determine the origin of uNGAL reciprocal cross-transplants of C3H/HeJ kidneys to C3H/HeOuJ bodies were performed, and vice versa. It was found that C3H/HeJ kidneys produce significantly less Ngal than C3H/HeOuJ animals ($p < 0.0005$). (b) *In situ* hybridization of these kidneys revealed a standard pattern of LPS induced Ngal expression in the distal epithelia of the kidney in the wild type kidney compared to the knockout kidney while Ngal mRNA was absent in the LPS-insensitive animal. These data confirmed that kidney epithelia derived Ngal is expressed in the TLR:Nf κ B pathway. (c) Investigation of the receptors involved in the UPEC activation of Ngal expression. C57BL/6, Tlr2, Tlr4, and Tlr11 knockout animals were challenged with an acute UTI (10-20 μ l of 5×10^9 CFU/ml CFT073). Tlr11 showed a similar phenotype to the Ngal KO.

[0016] Figure 5: Box plots of urinary neutrophil gelatinase-associated lipocalin (uNGAL) in patients with a UTI in multicenter study. (a) Patients presenting with gram negative UTIs had

abundant uNGAL compared to patients with gram positive infections (2078 ± 3215 vs 592 ± 1242 mg/g creatinine, $p=0.01$). One patient in the gram negative group had an uNGAL of 16,891 mg/g creatinine, not shown. (b) uNGAL parallel presence of urinary colony forming units (cfu). Patients with $>100,000$ cfu had significantly more uNGAL compared to patients with 10,000 and 100,000 cfu (2251 ± 3353 mg/g creatinine, 928 ± 1850 mg/g creatinine, $p=0.02$). Only one patient had $<10,000$ cfu of bacteria in their urine, and had an uNGAL level of 88 mg/g creatinine. One patient with more than 100,000 cfu had an uNGAL of 16,891 mg/g creatinine, not shown. (c) uNGAL concentration trended to increase with rising number of white blood cells. Patients with 11-20 and >30 cells per high powered field (hpf) had significantly more uNGAL compared to patients with 3-5 cells per hpf (1455 ± 1543 mg/g creatinine, 3023 ± 4067 mg/g creatinine, vs 219 ± 309 mg/g creatinine, $p=0.002$ and <0.001 , respectively). uNGAL levels were not significantly different between any other groups. uNGAL in patients with between 6 and 10 cells per hpf and those with between 21 and 30 cells hpf are 1365 ± 2531 mg/g creatinine and 1342 ± 2422 mg/g creatinine. One patient in the >30 cells group had an uNGAL of 16,891 mg/g creatinine, not shown.

[0017] Figure 6: NGAL is synthesized by bladder and kidney in a UTI. The distal nephron and the bladder epithelium responds to an uropathogenic infection by secreting uNGAL. (a, b) *In situ* hybridization demonstrated NGAL mRNA expression in the bladder epithelium (b) and in collecting ducts of the kidney (c) 1 d after an acute UTI ($10\text{--}20 \mu\text{l}$ of 5×10^9 CFU/ml CFT073). NGAL mRNA was predominantly expressed in the CD and was absent all other epithelial. (c) In order to determine the contribution of uNGAL from the kidney and the bladder, QPCR was done 1 d post-TU and Ngal copy number was determined. Almost 3 log orders greater Ngal copy number increase was seen in the kidney (left-hand column) compared to the bladder (right-hand column). (d) Primary culture of Ngal-Luc2/mC kidney medullary tissue and bladder tissue showing the responsiveness of uNGAL to a bacterial infection.

[0018] Figure 7: The kidney is the dominant source of urinary NGAL driven by TLR activation. Toll dependence of NGAL expression in Kidney. Previously it was observed that LPS-insensitive mice, C3HeJ, had significantly less uNGAL than wild type animals after i.p. challenge with LPS. To determine the origin of uNGAL reciprocal cross-transplants were performed of C3H/HeJ kidneys to C3H/HeOuJ bodies, and vice versa. It was found that C3H/HeJ kidneys produce significantly less Ngal than C3H/HeOuJ animals ($p < 0.0005$) (a). These data confirmed that Ngal is expressed in the TLR:Nf κ B pathway. (b) To further

investigate the receptors involved in the UPEC activation of Ngal expression during a UTI, C3HHeN control and C3HHeJ TLR4 mutant animals were challenged with an acute UTI (10-20 μ l of 5×10^9 CFU/ml CFT073). C3H/HeN animals had a lower urinary bacterial burden compared to C3H/HeJ mice. (c) We further found that C3H/HeJ kidney Ngal expression was significantly reduced (~20 fold) compared to C3H/HeN wild type animals. These results indicate that TLR4:Nf κ B pathway senses a UTI and activates uNGAL expression. (d) We generated a Ngal conditional KO in order to localize NGAL expressing cells during a UTI. By knocking out Ngal expression in the collecting ducts by breeding the HoxB7-cre recombinase animal with the NgallloxP/loxP animal, it was found that 1 day post-TU these animals had an intermediate phenotype to the global KO (EIIaCre) and the wild type in median CFU/ml urine.

DETAILED DESCRIPTION

[0019] The present invention is based, in part, on certain discoveries which are described more fully in the Examples section of the present application. For example, the present invention is based, in part, on the discovery that, in response to infection of the urinary tract with enterochelin-dependent uropathogenic bacteria, epithelial cells of the genitourinary tract secrete NGAL protein, which has bacteriostatic activity and inhibits growth of the bacteria. The present invention is also based, in part, on the elucidation of the biochemical pathways that result in the secretion of NGAL protein by the epithelial cells of the genitourinary tract in response to a urinary tract infection.

[0020] In some embodiments, the present invention provides methods for treating or preventing infection of the urinary tract or urosepsis in a subject, the methods comprising administering to the subject a therapeutically effective amount of one or more agents selected from the group consisting of: (a) an agent that stimulates genito-urinary tract epithelial cells of the subject to secrete NGAL protein, (b) NGAL, or a functional derivative thereof, and (c) combinations of one or more thereof. In other embodiments, the present invention provides methods for treating or preventing infection of the urinary tract or urosepsis in a subject, the methods comprising stimulating genito-urinary tract epithelial cells of the subject to secrete NGAL protein. In further embodiments, the present invention provides pharmaceutical compositions for use in treating a urinary tract infections and/or urosepsis. These and other aspects of the present invention are described in more detail in this "Detailed Description" section of the application, and also in the Summary of the Invention, Examples, Drawings, and Claims sections of the application.

[0021] Abbreviations and Definitions

[0022] The abbreviation “NGAL” refers to Neutrophil Gelatinase Associated Lipocalin. NGAL is also referred to in the art as human neutrophil lipocalin, siderocalin, α -microglobulin related protein, Scn-NGAL, lipocalin 2, 24p3, superinducible protein 24 (SIP24), uterocalin, and neu-related lipocalin. These alternative names for NGAL may be used interchangeably herein. Unless stated otherwise, the terms “NGAL” and “Ngal” as used herein, includes any NGAL protein, or functional derivative thereof. The terms “functional derivative” or “functional derivatives thereof,” as they are used herein in relation to NGAL, refer to any fragments, variants, mutants or analogs of NGAL that retain the ability to bind to iron, including, but not limited to iron bound to siderophores, and/or retain bacteriostatic activity. Functional derivative of NGAL include, but are not limited to, mutated versions of the NGAL protein, and chemically modified versions of the NGAL protein. Such functional derivatives may have one or more amino acids or other chemical moieties added, removed, or substituted. The term “analog” includes structural equivalent or mimetics, as understood by those of skill in the art. In some embodiments the NGAL protein is wild-type NGAL, such as wild-type human NGAL. In some contexts, the term NGAL may also be used herein to refer to a nucleotide that encodes an NGAL protein, or a functional derivative thereof, such as a DNA or RNA molecule that encodes an NGAL protein.

[0023] The abbreviation “uNGAL” is an abbreviation for urinary NGAL and refers to NGAL that is found in the urine or elsewhere in the genito-urinary tract, or that is expressed by cells of the genito-urinary tract.

[0024] The abbreviation “UTI” refers to a urinary tract infection.

[0025] The abbreviation “UPEC” refers to a uropathogenic *Eschericia coli* – a type of bacterium.

[0026] The abbreviation “*E. coli*” refers to a the bacterium *Eschericia coli*.

[0027] The abbreviation “CFU” refers to colony-forming units, for example of a bacterium. The abbreviation “uCFU” refers to urinary colony-forming units, for example of a urinary or urinary tract bacterium.

[0028] The abbreviation “TU” refers to trans-urethral.

[0029] The abbreviations “IP” and “i.p.” refer to intraperitoneal.

[0030] The abbreviation “KO” refers to knock-out or knock-out organism (e.g. mouse).

[0031] The abbreviation “CKO” refers to conditional knock-out or conditional knock-out organism (e.g. mouse).

[0032] The abbreviation “WT” refers to wild-type.

[0033] The abbreviation “GU” refers to genitor-urinary.

[0034] The abbreviation “CD” refers to the collecting duct of the kidney.

[0035] The abbreviation “TAL” refers to the tall ascending limb of Henlé in the kidney.

[0036] The abbreviation “GFR” refers to the glomerular filtration rate of the kidney.

[0037] The abbreviation “PCR” refers to polymerase chain reaction.

[0038] The abbreviation “QPCR” refers to a quantitative polymerase chain reaction.

[0039] The term “urosepsis” is used herein in accordance with its normal meaning in clinical medicine, and refers to bacteremia that is secondary to a UTI.

[0040] The abbreviation “AKI” refers to acute kidney injury.

[0041] The abbreviation “NRF2” refers to nuclear factor (erythroid-derived 2)-like 2, which is also known as NFE2L2 and Nrf2.

[0042] The abbreviation “HIF” refers to hypoxia inducible factor.

[0043] The abbreviation “NF- κ B” refers to nuclear factor kappa-light-chain-enhancer of activated B cells.

[0044] The phrases "pharmaceutically acceptable," "pharmacologically acceptable," and "physiologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human.

[0045] The phrase "pharmaceutically acceptable carrier" as used herein means a material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, that can be used in a composition of the invention without adversely affecting the biological activity of the active ingredient(s) of the compositions, such as NGAL. Each carrier should be "acceptable" in the sense of being compatible with other ingredients of the composition, including the active ingredients, such as NGAL, and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include, but are not limited to: any and all solvents, dispersion media,

coatings, surfactants, antioxidants, preservatives, isotonic agents, absorption delaying agents, salts, preservatives, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art. Except insofar as any conventional carrier is incompatible with the active ingredients of the compositions described herein, such as NGAL, its use in the therapeutic or pharmaceutical compositions is contemplated.

[0046] The phrase "therapeutically effective amount" refers to an amount of the active ingredient of a compositions described herein, such as NGAL, that is effective to produce beneficial results, particularly with respect to the treatment, prevention or amelioration of UTI or urosepsis, as described herein, in the recipient, such as an animal or a human patient. Such amounts can readily be determined by those of ordinary skill in the art, for example on the basis of published literature, *in vitro* testing, or by conducting studies in animals or in human subjects.

[0047] A "patient", "recipient", or "subject" means an animal or organism, such as a warm-blooded animal or organism. Illustrative animals include, without limitation, mammals, for example, humans, non-human primates, pigs, cats, dogs, rodents, horses, cattle, sheep, goats and cows. The invention is particularly suitable for human patients and subjects.

[0048] The words "a" and "an" as used herein refers to "one or more". More specifically, the use of "comprising," "having," or other open language in claims that claim a combination or method employing an object, denotes that "one or more of the object" can be employed in the claimed method or combination.

[0049] As used herein the term "about" is used herein to mean approximately, roughly, around, or in the region of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20 percent up or down (higher or lower).

[0050] NGAL

[0051] NGAL protein, or functional derivatives thereof, can be manufactured by any suitable method known in the art for manufacture of protein drugs. For example NGAL protein can be made using standard techniques known for the production of recombinant proteins, for example by delivering to a cell, such as a bacterial cell or a mammalian cell, an

expression vector containing a nucleotide sequence that encodes an NGAL protein under the control of a suitable promoter, and culturing the cell under conditions in which the protein will be expressed. Nucleotide sequences that encode NGAL proteins are well known in the art. Methods for the large scale culture, isolation, and purification of recombinant proteins are well known in the art and can be used in the manufacture of the NGAL proteins of the present invention. Similarly, methods of producing peptides and proteins synthetically are known in the art and can be used in the manufacture of the NGAL proteins of the present invention.

[0052] In certain embodiments, the NGAL proteins, or functional derivatives thereof, may be used as fusion proteins comprising the NGAL protein and one or more additional "tags." Such additional tags can be fused to the N- or C-terminus of the NGAL proteins, or can in some instances be added at an internal location to the extent that the inclusion of the tag does not adversely affect the function of the NGAL protein. Suitable tags include, but are not limited to glutathione-S-transferase (GST), poly-histidine (His), alkaline phosphatase (AP), horseradish peroxidase (HRP), and green fluorescent protein (GFP). Other suitable tags will also be apparent to those skilled in the art. The tags may be useful for several applications, including to assist in the isolation and/or purification of the NGAL proteins and/or to facilitate their detection.

[0053] Many chemical modifications of proteins are known in the art to be useful for improving the properties of protein-based drugs and such modifications can be used in accordance with the present invention to improve the stability and reduce the immunogenicity of the NGAL proteins of the invention for therapeutic applications. For example, it is well known in the art that the process of covalent attachment of polyethylene glycol polymer chains to another molecule (i.e. PEGylation) can "mask" a proteinaceous agent from the host's immune system, and also increase the hydrodynamic size (size in solution), prolong the circulatory half-life, and improve water solubility of protein-based drugs. Various other chemical modifications are also known and used in the art and can be used in conjunction with the NGAL proteins of the invention.

[0054] In some embodiments, it may also be desirable to use a complex containing an NGAL protein and a siderophore, such as enterochelin, or a derivative or variant thereof. Such complexes can readily be prepared using standard methodologies known in the art. For example, an NGAL-siderophore complex can be prepared by mixing NGAL and a siderophore together in a molar ratio of 1:1 (e.g. enterochelin) or 1:3 (e.g. catechol). The

mixture can be incubated at room temperature for a suitable time, e.g. 30 minutes, to allow for complex formation. Unbound siderophore can then be removed / separated from the bound siderophore-NGAL complexes using standard separation techniques, such as centrifugation based techniques, filter-based techniques, or other size-based separation techniques. Siderophores that are known in the art include, but are not limited to enterochelin, TRENCAM, MECAM, TRENCAM-3,2-HOPO, parabactin, carboxymycobactin, fusigen, triacetylfusarinine, ferriochrome, coprogen, rhodotorulic acid, ornibactin, exochelin, ferrioxamine, desferrioxamine B, aerobactin, ferrichrome, rhizoferrin, pyochelin, pyoverdine. The structures of these compounds are disclosed in Holmes et al., Structure, 2005, 13:29-41 and Flo et al., Nature, 2004, 432: 917-921, the contents of which are hereby incorporated by reference. Several of the above siderophores are known to bind to lipocalins, including NGAL, and complexes of these siderophores and lipocalins are known to be able to sequester iron (see for example, Holmes et al., Structure, 2005, 13:29-41 and Flo et al., Nature, 2004, 432: 917-921; Goetz et al, Molecular Cell, 2002, 10: 1033-1043 and Mori, et al., "Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury." J. Clin Invest., 2005, 115, 610-621). Thus, in some aspects the present invention contemplates the use and/or administration of an NGAL protein together with a siderophore, including, but not limited to, the siderophores listed herein. In preferred aspects the siderophore is selected from the group consisting of enterochelin, pyrogallol, carboxymycobactin, catechol, and variants or derivatives thereof. Any variant or derivative of such siderophores that retains the ability to bind to iron (ideally in a pH insensitive manner) and that retains the ability to bind to NGAL may be used in accordance with the present invention.

[0055] Agents that Stimulate Production of NGAL by GU Tract Epithelial Cells

[0056] In some embodiments, the present invention provides methods for treating or preventing UTI or urosepsis in a subject comprising administering to the subject an agent that stimulates production of NGAL by epithelial cells of the urinary tract. In other embodiment, the present invention provides compositions that comprise an agent that stimulates production of NGAL by epithelial cells of the urinary tract. Any suitable agent that stimulates the production of NGAL by epithelial cells of the urinary tract may be used in the methods of compositions of the invention.

[0057] In some embodiments, the present invention provides methods for treatment or prevention of UTI or urosepsis that comprise administration of an NFκB activator. In other

embodiments, the present invention provides compositions that comprise an NF κ B activator. Any NF κ B modulator that stimulates the expression and/or secretion of NGAL by epithelial cells of the urinary tract can be used in accordance with the present invention, including, but not limited to compounds SRI#22771, SRI#22772, SRI#22773, SRI#22774, SRI#22775, SRI#22776, SRI#22777, SRI#22778, SRI#22779, SRI#22780, SRI#22781, SRI#22782, SRI#22816, SRI#22817, SRI#22818, SRI#22819, SRI#22820, and SRI#22864, as described in Manuvakhova et al., Identification of Novel Small Molecule Activators of Nuclear Factor- κ B With Neuroprotective Action Via High-Throughput Screening, *Journal of Neuroscience Research*, 89:58–72 (2011), the contents of which are hereby incorporated by reference.

[0058] In some embodiments, the present invention provides methods for treatment or prevention of UTI or urosepsis that comprise administration of an activator of a TLR-NF κ B pathway, such as the TLR4-NF κ B pathway or the TLR11-NF κ B pathway. In other embodiments, the present invention provides compositions that comprise an an activator of a TLR-NF κ B pathway, such as the TLR4-NF κ B pathway or the TLR11-NF κ B pathway. Any activator of a TLR-NF κ B pathway modulator that stimulates the expression and/or secretion of NGAL by epithelial cells of the urinary tract can be used in accordance with the present invention, including, but not limited to NF κ B, and the TLR4 activators described in Huang et al., “Synthesis of serine-based glycolipids as potential TLR4 activators,” *Org. Biomol. Chem.*, 2011, 9, 2492-2504, the contents of which are hereby incorporated by reference.

[0059] In some embodiments, the present invention provides methods for treatment or prevention of UTI or urosepsis that comprise administration of a lipid A derivative, an endotoxin derivative, or a lipopolysaccharide derivative. In other embodiments, the present invention provides compositions that comprise a lipid A derivative, an endotoxin derivative or a lipopolysaccharide derivative. Any lipid A derivative, endotoxin derivative, or lipopolysaccharide derivative that stimulates the expression and/or secretion of NGAL by epithelial cells of the urinary tract, and is suitable for clinical use (for example, it is not toxic) can be used in accordance with the present invention, including, but not limited to, monophosphoryl Lipid A (as described in Johnson et al., “Characterization of a nontoxic monophosphoryl lipid A,” *Rev. Infect. Dis.* (1987), 9 Suppl 5:S512-6), E5531 (as described in Kawata et al., “A synthetic non-toxic lipid A derivative blocks the immunobiological activities of lipopolysaccharide.” *Br J Pharmacol.* 1999 Jun;127(4):853-62), the lipid A derivative described in Santhanam et al., (“Preparation of a Lipid A Derivative That Contains

a 27-Hydroxyoctacosanoic Acid Moiety,” Org. Lett., 2004, 6 (19), pp 3333–3336), and Lipid X, Lipid Y, “incomplete lipid A,” or monophosphoryl lipid A (TLC-3) (as described in Takayama et al.,” Separation and characterization of toxic and nontoxic forms of lipid A,” Reviews of Infectious Diseases (1984), 6(4): 439-43), the contents of each of which are hereby incorporated by reference in their entireties.

[0060] In some embodiments, the present invention provides methods for treatment or prevention of UTI or urosepsis that comprise administration of a HIF modulator. In other embodiments, the present invention provides compositions that comprise a HIF modulator. Any HIF modulator that stimulates the expression and/or secretion of NGAL by epithelial cells of the urinary tract can be used in accordance with the present invention, including, but not limited to, HIF, HIF prolyl-hydroxylase inhibitors, such as FG-2216 and FG-4592, (See, Bruegge K, Jelkmann W, Metzen E (2007). "Hydroxylation of hypoxia-inducible transcription factors and chemical compounds targeting the HIF-alpha hydroxylases". Curr. Med. Chem. 14 (17), the contents of which are hereby incorporated by reference in its entirety), deferoxamine, desferoxamine mesylate, Desferal Mesylate®, desferri-exochelin, ciclopirox olamine [3, Loprox®, 6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridone 2-aminoethanol], 8-methyl-pyridoxatin, N-oxaloylglycine (NOG), DMOG (6, dimethyl-oxalylglycine), 3,4-dihydroxybenzoate, or pyridine-2,5-dicarboxylate. Other HIF modulators are described in Nagle et al., Curr. Pharm. Des. 2006; 12(21): 2673–2688, and Semenzathe et al., Drug Discovery Today, 2007, 12(19-20): 853-859, the contents of which are hereby incorporated by reference.

[0061] In some embodiments, the present invention provides methods for treatment or prevention of UTI or urosepsis that comprise administration of an NRF2 modulator. In other embodiments, the present invention provides compositions that comprise an NRF2 modulator. Any NRF2 modulator that stimulates the expression and/or secretion of NGAL by epithelial cells of the urinary tract can be used in accordance with the present invention, including, but not limited to, dithiolethione NRF2 modulators, oltipraz, olean triterpenoid compounds, bardoxolone methyl, and resveratrol.

[0062] Urinary Tract Infections & Urosepsis

[0063] In some embodiments, the present invention provides compositions and methods for the treatment or prevention of UTI or urosepsis. In some embodiments, the UTI or urosepsis is caused by, or associated with, one or more bacterial species. In some

embodiments, the UTI or urosepsis is caused by, or associated with, one or more siderophore-dependent uropathogenic bacteria, such as catecholate-dependent uropathogenic bacteria. In some embodiments, the UTI or urosepsis is caused by, or associated with, one or more enterochelin-dependent uropathogenic bacteria. In some embodiments, the UTI or urosepsis is caused by, or associated with, an E.coli infection.

[0064] Pharmaceutical Compositions & Administration

[0065] In some embodiments, the present invention provides pharmaceutical compositions for use in treating or preventing a urinary tract infection or urosepsis. Such compositions comprising a therapeutically effective amount of an agent that stimulates genito-urinary tract epithelial cells to secrete NGAL protein, and/or a therapeutically effective amount of NGAL, or a functional derivative thereof. Examples of agents that stimulate genito-urinary tract epithelial cells to secrete NGAL protein include, but are not limited to derivatives of lipid A, derivatives of lipopolysaccharide, derivatives of endotoxin, activators of the TLR4-NFκB pathway, activators of the TLR11-NFκB pathway, activators of NFκB, NRF2 modulators, and HIF modulators. Each of these agents (including NGAL, or derivatives thereof), may be formulated into a pharmaceutical composition.

[0066] The pharmaceutical compositions of the invention include those suitable for oral or parenteral (including intramuscular, subcutaneous and intravenous) administration. Administration of a therapeutically effective amount of any of the agents described herein can be accomplished via any mode of administration suitable for therapeutic agents. One of skill in the art can readily select a suitable mode of administration without undue experimentation. Suitable modes may include systemic or local administration such as oral, nasal, parenteral, transdermal, subcutaneous, topical, intravenous (both bolus and infusion), intraperitoneal, or intramuscular administration modes. In some embodiments, oral or intravenous administration is used. In other embodiments, the compositions of the invention are administered directly to the desired site of action, such as for example, the urinary tract, for example by local injection or local infusion or by use of (e.g. conjugation to) agents useful for targeting proteins or pharmaceuticals to specific tissues, such as antibodies etc. In some embodiments, the compositions of the invention are administered directly to the kidney or elsewhere in the genitourinary tract, for example by transurethral (TU) delivery. In some embodiments the compositions of the invention are administered directly to the genitourinary tract using a catheter or similar medical device. For example, in cases where a catheter is to be inserted into a subject transurethrally, for example in the course of a medical procedure, it

may be desirable to deliver the compositions of the invention transurethraly prophylactically to the subject, to prevent or mitigate the effects of any UTI that could otherwise be caused as a result of the medical procedure.

[0067] Depending on the intended mode of administration, the agents of the invention may be in solid, semi-solid or liquid dosage form, such as, for example, injectables, tablets, suppositories, pills, time-release capsules, elixirs, tinctures, emulsions, syrups, powders, liquids, gels, creams, suspensions, or the like. In one embodiment the agents of the invention may be formulated in unit dosage forms, consistent with conventional pharmaceutical practices. Liquid, particularly injectable, compositions can, for example, be prepared by dissolution or dispersion. For example, agents of the invention can be admixed with a pharmaceutically acceptable solvent such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form an injectable isotonic solution or suspension.

[0068] Parental injectable administration can be used for subcutaneous, intramuscular or intravenous injections and infusions. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions or solid forms suitable for dissolving in liquid prior to injection. One embodiment, for parenteral administration, employs the implantation of a slow-release or sustained-released system, according to U.S. Pat. No. 3,710,795, incorporated herein by reference.

[0069] Compositions comprising the agents of the invention can be sterilized and may contain any suitable adjuvants, preservatives, stabilizers, wetting agents, emulsifying agents, solution promoters, salts (e.g. for regulating the osmotic pressure), pH buffering agents, and/or other pharmaceutically acceptable substances, including, but not limited to, sodium acetate or triethanolamine oleate. In addition, the compositions of the invention may also contain other therapeutically useful substances, such as, for example, other iron chelators or other bacteriostatic or antibacterial agents. In some embodiment, the compositions of the invention may comprise one or more additional agents that are useful for the treatment or prevention of UTI or urosepsis, such as bacteriostatic agents and antibiotics that are useful in the treatment of UTI or urosepsis. For example, such an additional agent may be a bacteriostatic agent or antibiotics that is effective to inhibit the growth of uropathogenic E.coli (UPEC) strains, including enterochelin-dependent UPECs.

[0070] The methods of treatment provided herein may also comprise treatment with a bacteriostatic agent and/or antibiotic that is useful in the treatment of UTI or urosepsis – in

addition to: (a) NGAL, or a functional derivative thereof, or (b) an agent that stimulates the production of NGAL by urinary tract epithelial cells, or (c) any combination thereof. For example, such an additional agent may be a bacteriostatic agent or antibiotic that is effective to inhibit the growth of uropathogenic E.coli (UPEC) strains, including enterochelin-dependent UPECs.

[0071] The compositions of the invention can be prepared according to conventional mixing, granulating or coating methods, respectively, and the present pharmaceutical compositions can contain from about 0.1% to about 99%, preferably from about 1% to about 70% of the composition of the invention by weight or volume.

[0072] The dose and dosage regimen to be used in accordance with the methods of treatment of the invention can be determined in accordance with a variety of factors including the species, age, weight, sex and medical condition of the subject; the severity of the condition; the route of administration; and the renal or hepatic function of the subject. A person skilled in the art can readily determine and/or prescribe an effective amount of an agent of the invention useful for treating or preventing UTI or urosepsis, for example, taking into account the factors described above. Dosage strategies are also provided in L.S. Goodman, et al., *The Pharmacological Basis of Therapeutics*, 201-26 (5th ed.1975), which is herein incorporated by reference in its entirety. In one embodiment, compositions of the invention are administered such that the active agent(s) is administered at a dose range of about 1 to about 100 mg/kg body weight, and typically at a dosage of about 1 to about 10 mg/kg body weight, or is administered at a dose that results in a concentration in the range of about 0.1 ng/ml to about 100 ng/ml, e.g., in the range of about 1.0 ng/ml to about 20 ng/ml, in the blood or locally at the site of action, such as in the urinary tract.

[0073] **Screening Methods**

[0074] The present invention provides methods of screening for agents that stimulate epithelial cells of the urinary tract, such as kidney epithelial cells (including epithelial cells of the collecting ducts or of the thick ascending limb of Henlé), bladder epithelial cells, and urethral epithelial cells, to produce NGAL mRNA or protein. In some embodiments, such screening methods comprise providing a population of urinary tract epithelial cells, contacting the population of urinary tract epithelial cells with one or more test agents, and testing for production of NGAL mRNA or protein by the urinary tract epithelial cells, thereby identifying agents that stimulate production of NGAL mRNA or protein by the urinary tract

epithelial cells. In one embodiment, the present invention provides a method of identifying an agent that stimulates epithelial cells of the urinary tract to produce NGAL mRNA or NGAL protein, the method comprising: (a) providing a test population of urinary tract epithelial cells and a control population of urinary tract epithelial cells, (b) contacting the test population of urinary tract epithelial cells with one or more test agents, (c) contacting the control population of urinary tract epithelial cells with no agent or with one or more negative control agents, and (d) determining the level of NGAL mRNA or NGAL protein in the test population and the control population, or in a culture supernatant thereof, wherein a level of NGAL mRNA or NGAL protein in the test population, or a culture supernatant thereof, that is higher than the level of NGAL mRNA or NGAL protein in the control population, or a culture supernatant thereof, indicates that the test agent is an agent that stimulates production of NGAL mRNA or NGAL protein by the urinary tract epithelial cells. In another embodiment, the present invention provides a method of identifying an agent that stimulates epithelial cells of the urinary tract to produce NGAL mRNA or NGAL protein, the method comprising: (a) providing a population of urinary tract epithelial cells, (b) determining the control level of NGAL mRNA or NGAL protein in the population of urinary tract epithelial cells, or in a culture supernatant thereof, wherein the control level is the level of NGAL mRNA or NGAL protein present prior to contacting the urinary tract epithelial cells with one or more test agents, (c) contacting the urinary tract epithelial cells with one or more test agents, (d) determining the test level of NGAL mRNA or NGAL protein in the population of urinary tract epithelial cells, or in a culture supernatant thereof, wherein the test level is the level of NGAL mRNA or NGAL protein present subsequent to contacting the urinary tract epithelial cells with the one or more test agents, wherein if the test level of NGAL mRNA or NGAL protein exceeds the control level of NGAL mRNA or NGAL protein, the test agent is an agent that stimulates production of NGAL mRNA or NGAL protein by the urinary tract epithelial cells.

[0075] In some such embodiments, the urinary tract epithelial cells may be *in vivo*, for example in a mouse model. In other embodiments, the urinary tract epithelial cells may be cultured *in vitro*. Urinary tract epithelial cells that are cultured *in vitro* may be primary cultures, or may be derived from primary cultures, or may be cell lines, such as established urinary tract epithelial cell lines, including kidney epithelial cell lines, bladder epithelial cells lines, urethral epithelial cell lines, and the like. The test agents may be any suitable test agents, including, but not limited to, libraries of small molecule drugs, libraries of

proteinaceous or peptide drugs (including peptidomimetic drugs), libraries of antibodies, libraries of RNA molecules (including, but not limited to, antisense RNAs, siRNAs, shRNAs, and microRNAs, ribozymes), and the like. In addition to libraries of test agents, individual test agents, or smaller populations of test agents, may also be used. Any suitable negative controls can be used. For example, the epithelial cells may be contacted with no test agent, or with an inactive agent, such as an agent that is known not to stimulate NGAL production. Any suitable positive control may be used. For example, an agent that is known to stimulate production of NGAL by urinary tract epithelial cells, such as, for example, Lipid A. Any suitable means may be used to detect NGAL production by the urinary tract epithelial cells. In one embodiment, secreted NGAL protein is detected in cell supernatants. In another embodiment, NGAL protein within the epithelial cells is detected. NGAL protein may be detected using any suitable means. In one embodiment, NGAL protein is detected using an antibody to NGAL. The NGAL antibody may be labeled with a detectable moiety, or a secondary antibody that is labeled with a detectable moiety may be used. Suitable detectable moieties may include enzyme substrates (such as horseradish peroxidase, alkaline phosphatase, and the like), and fluorescent labels (such as green fluorescent protein, and the like). In one embodiment NGAL protein may be detected in an ELISA assay using an anti-NGAL antibody. In another embodiment NGAL mRNA is detected. NGAL mRNA may be detected using any suitable means, including, but not limited to, *in situ* hybridization, Northern blotting, PCR, QPCR, and the like. Any suitable probes or primers for detection of NGAL mRNA may be used.

[0076] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be within the scope of the present invention.

[0077] The invention is further described by the following non-limiting Examples.

EXAMPLES

EXAMPLE 1

[0078] The numbers in superscript below refer to the corresponding numbered reference(s) at the end of this Example.

[0079] UTIs are one of the most prevalent and resource taxing diseases with 13.3% (12.8 million) of all women and 2.3%(2 million) of men in the USA are infected annually producing an annual cost of \$3.5 billion for evaluation and treatment¹. In 2000 there were an estimated 11.02 million visits (2.05 million men; 8.97 million women) to a physician's office or hospital with UTI listed as any diagnosis.¹ Uropathogenic *E. coli* (UPEC) represent 70-95% of all cases of UTI and many of these bacteria rely on catecholate-siderophores as their primary iron uptake mechanism.¹ Urine dipsticks are used to read the biochemical signature of a UTI. Dipsticks recognize the presence of leukocyte esterase and nitrite in the urine. Leukocyte esterase corresponds to pyuria and nitrite reflects the presence of Enterobacteriaceae, which convert urinary nitrate to nitrite.^{3,4} In a review of six studies including women aged 17 to 70 with suspected UTI in primary care settings, positive dipstick findings (nitrite or leukocyte esterase and blood) had sensitivity and specificity of 75 and 66 percent, respectively⁵ and in children they are at best 88 percent sensitive.⁶

[0080] Neutrophil Gelatinase Associated Lipocalin (NGAL) is a secreted lipocalin which is markedly upregulated and expressed by the kidney human adults⁷ and children⁸, as well as in mice⁹⁻¹¹, rats, and pigs in proportion to the dose of stimuli such as ischemia-reperfusion (I/R)⁹, hypoxia, drug toxicity, and bacterial infection¹⁰⁻¹³ which typically generate kidney damage. However it has not been clear why this protein is expressed in the urinary system after kidney damage of different types. NGAL is a bacteriostatic protein¹⁴ by binding catecholate-siderophore¹⁵ which sequesters iron from bacteria. The studies described in this Example relate to the relationship between NGAL expression by the kidney and lower urinary tract infection.

[0081] To investigate the relationship between NGAL and UPEC a pyelonephritogenic clinical isolate of uropathogenic *Escherichia coli*, CFT073, was aliquoted in 96 well plates containing M9 minimal medium supplemented with MgCl₂ and glucose (see green hashed line: Fig. 1a), and bacterial growth was monitored by spectrophotometry. Bacterial growth was significantly inhibited (Fig. 1a) when the UPEC was grown with the addition of mouse (m) NGAL (red line and open red squares: 5μM), but it could be rescued by the addition of FeCl₃ (blue line with open blue circles: 1mM). This data is consistent with NGAL's activity in iron scavenging.¹⁵

[0082] To determine the role NGAL has in an acute UTI *in vivo* a conditional NGALloxP/loxP animal and tissue specific knockouts were generated (Methods and Fig. 2). The NGALloxP/loxP mouse was bred with EIIa-Cre16 mouse (NGALloxP/loxP, EIIa-Cre)

which generated a knock-out of NGAL in all cells. NGAL wild-type (Ngal^{+/+}) mice (white bar) and NgalloxP/loxP, EIIa-Cre (black bars) mice were challenged with a transurethral (TU) infection of the UPEC (10-20 μ l of 5×10^9 CFU/ml CFT073) and urinary (u) NGAL and urinary colony forming units (uCFU) were monitored for one week (Fig. 1b). In Ngal^{+/+} mice we noted that the secretion of uNGAL mirrored both the onset of the urinary tract infection (UTI), indicated by log order increases in uCFU, and the resolution of the acute phase of the infection (~day 3-4, Fig. 1b). NGAL^{+/+} animals with the acute UTI cleared the CFT073 bacteria within 3-5 days after the initial challenge with the UPEC, but animals without a functioning NGAL allele took significantly more time (6 days) than the wild type mice to clear the UPEC (NgalloxP/loxP, EIIa-Cre mice had a log order more uCFU than Ngal^{+/+} at 2, 3, 4 and 5 days post-TU, $p < 0.05$, UTest: Fig. 1b). Hence, uNGAL is necessary to rapidly clear an acute UTI by an enterochelin dependent UPEC and the NGAL protein itself is sufficient for bacteriostasis.

[0083] To determine the normal cellular source of uNGAL in response an acute UPEC infection, *in situ* hybridization was performed in NGAL^{+/+} kidneys 1 day post-TU injection of the UPEC (5×10^9 CFU/ml). High levels of NGAL RNA were expressed by bladder epithelium (Fig. 3a) and through microscopic examination of the kidney, NGAL mRNA was identified in the epithelia of the Thick Ascending Limb of Henle (TAL) and the Collecting Ducts (CD)(Fig. 3b). To determine the relative contribution of these organs to uNGAL production, QPCR was performed and the copy number of NGAL per organ 1 day post-TU challenge with the UPEC was determined. IP injection was used as a control to stimulate NGAL expression. With either systemic or transurethral application of bacteria, the kidney was the major contributor to uNGAL as measured by the number of copies of NGAL respective of total RNA (Fig. 3c).

[0084] The data showed that both the kidney and the bladder might contribute to the uNGAL pool. NGAL expression was further dissected by selectively knocking out NGAL in different segments of the GU tract. NGAL was first knocked out in the collecting duct (CD) by generating a NGALloxP/loxP, HoxB7/cre19 (NgalloxP/loxP, HoxB7/cre) CKO mouse. HoxB7/cre has been noted to be expressed in the ureteric epithelium of the distal, non-branching medullary collecting ducts and the epithelium of the ureter¹⁹. It was found that the HoxB7 compartment was a major contributor to uNGAL because there was greater than a log order increase in median uCFU after a TU challenge with the UPEC compared to the wild type mice (Fig. 4). These data support findings that NGAL expression was localized after

urinary infection and was localized to the collecting duct⁹. Therefore many types of GU epithelia generate uNGAL protein. The combination of *in situ* hybridization, organ copy number analysis, and segment specific knockouts indicated *in vivo* that the major source of NGAL RNA is the kidney and the major source of the NGAL protein is also the kidney.

[0085] Previous findings⁹ from isolated primary cells revealed that bacterial gram-negative components, such as lipid A, bind to Toll-like receptors (TLRs), such as Toll-like receptor 4 (TLR4), and activate NF- κ B⁸ which induces NGAL *in vitro*. To determine whether uNGAL originated from the kidney in a TLR-dependent fashion, kidney transplantation between Tlr4-mutant CH3/HeJ mice and wild type CH3/HeOuJ mice was performed. To evaluate the contribution of TLR signaling to NGAL expression a cross-transplant model using TLR mutants and systemic administration of LPS as a positive control was used. The CH3/HeJ kidneys from the LPS-insensitive mice were transplanted into CH3/HeOuJ LPS-sensitive (control) mouse bodies, and vice versa. Two weeks after graft maturation, when uNGAL and sCr had stabilized to normal values, a low dose of lipid A (1 mg/kg of body weight) was administered to induce Ngal expression in the kidney (Fig. 4a). QPCR revealed that the wild type kidney in the Tlr4-mutant body had a 15.6 ± 2.3 fold increase in Ngal expression while the Tlr4-mutant kidney in the wild type body had only a 4.5 ± 2.6 fold increase in Ngal expression. It is not surprising that there was some Ngal induction in the knockout (KO) kidney because CH3/HeJ are partial KOs and it has also been previously shown that lipid A can signal through the MyD88-dependent pathway without a functioning Tlr4. These results suggests that TLR4 is the receptor to lipid A in the kidney, and indicate that NGAL is induced by LPS activation of the TLR4::NfKB pathway.

[0086] The kidney can also sense a bacterial infection by the presence of necrotic cell debris from endogenous and endogenous origin. It has been shown that TLR4 is activated by heat-shock proteins, fibronectin, hyaluronic acid, heparin sulfate and fibrinogen,²⁰⁻²⁶ suggesting that the kidney can gauge the early onset of a bacterial infection in the blood and the bladder. TLR4 can bind to a myriad of factors, and this single-pass transmembrane receptor can elicit a tightly regulated signal transduction pathway from various molecules.

[0087] Many of the toll-like receptors are expressed in the kidney epithelium,²⁷ thus to determine which TLR is responsible for inducing NGAL expression in response to a UTI TLR2, TLR4 and TLR11 were challenged with TU injection of CFT073 (Fig 7(c)). TLR2 and TLR4 are the most expressed in distal and proximal tubules and Bowman's capsule.²⁸ During sepsis and ischemia, both TLR2 and TLR4 are highly upregulated and their spatial

distribution changes according to the event. Furthermore immunohistological localization reveals TLR2 and TLR4 in the apical membrane of the proximal tubule.²⁹ TLR11 has been shown to be expressed in the collecting duct and in the bladder epithelium and responds to UPECs.³⁰ However, a difference was observed in uCFU as early as a day after infection with the TLR11 mutant compared to its wild-type littermate (Fig. 7(c)). No differences in uCFUs from the TLR2 and TLR4 mutants were seen. A recent functional genomic study showed that C3H/HeJ bladders markedly expressed Ngal by gene arrays from laser capture microdissected urothelial cells.³¹ Furthermore, CFT073 has been observed to subvert TLR signaling via MyD88-dependent by secreting a TIR-domain protein that may bind directly to MyD88.³² Therefore, CFT073 may induce NGAL expression in the kidney through TLR11 via a MyD88-independent activation of NF- κ B.

[0088] The results described in this Example show that kidney epithelia produce NGAL, a bacteriostatic molecule, which can inhibit the growth of a highly pathogenic strain of uropathogenic bacteria (CFT073). NGAL is a secreted molecule shown to be a kidney growth factor,^{33,34} and has been shown to have a high affinity for iron bound catecholate-siderophores¹⁵ and endogenous iron bound catechol,³⁵ thus making it a potent antimicrobial by limiting *Escherichia coli*'s¹⁴ access to iron. However its role in urinary tract infections (UTI) was previously unknown. These data establish a rationale for the abundant NGAL secretion from the kidney in both aseptic and septic states in which the GU is part of the innate immune defense pathway and its expression is either prophylactic against a potential bacterial infection during an injury or protective against a current bacterial invasion. The results presented here show that NGAL is secreted in response to highly pathogenic strain of uropathogenic *E. coli* into the urinary space by the kidney epithelia⁹ and that signaling through TLR11 can inhibit bacterial growth.

[0089] These studies show that the kidney secretes NGAL in response to an impending bacterial infection. Perhaps the kidney is being primed for bacterial invasion due to the sharp reduction in glomerular filtration rate (urine flow). GFP reduction can occur from cast formation due to ischemia, toxic injury, and inflammation to the kidney while a reduction in urine flow can occur in injuries to the bladder such as obstruction and cancer. It is plausible that this reduction in urine flow from renal damage would make the kidney more vulnerable to bacterial invasion and thus pyelonephritis. Therefore, the kidney expresses a bacteriostatic molecule as a preemptive step to suppress an ascending UPEC from entering the kidney and subsequently entering the bloodstream.

[0090] Materials And Methods

[0091] Mouse husbandry. NgallloxP/loxP, NgaliEII-Cre, NgaliHoxB7-cre, C57BL6, C3H/HeJ, C3H/HeOuJ, and Tlr11 mice were raised and experimentally used in this study.

[0092] Ngali Cre-lox Targeting Construct Generation. The BAC clone was made recombinogenic by transformation with a plasmid from the Red/ET cloning kit (Gene Bridges, Heidelberg, Germany) and the homology domains were subcloned into a backbone vector by a homologous recombination based Red/ET cloning method. A loxP site was inserted into intron 1, in a 2-step procedure. A loxP-flanked neo selection marker cassette (loxP-neo-loxP) is inserted by homologous recombination and then Cre is expressed in bacterial cells (EL350) to recombine the loxP sites and excise the selection marker, leaving a single loxP site. A neo cassette is inserted in a 2-step procedure into intron 5 using homologous recombination to insert a unique restriction site (Bsiw I) and then to ligate a neo cassette by conventional methods.

[0093] Electroporation into ES cells. The targeting vector was linearized, electroporated and clones selected with neo. Primers, A1, 2, 3 were 3' of the short homology arm (SA) outside the region used to generate the targeting construct and N1 was located at the 5' end of the Neo cassette amplify 2.3, 2.4, and 2.4kb fragments respectively. Control PCR used T1 and T2, which are inside the targeting construct.

[0094] Excision of neo gene. F0 mice derived from ES cells are crossed with a ubiquitous FLP deleter (including germ cells) under the control of human ACTB (β actin) promoter (B6;SJLTg(ACTFLPe)9205Dym/J (JAX® Mice Stock # 003800)).³⁶

[0095] The efficiency of FLP-excision of FRT-flanked DNA sequence was reported to 100% in F1 mice (heterozygote β actin-FLPe X heterozygote FRT-disrupted lacZ reporter gene driven by HMGC α reductase promoter/enhancer sequence). Genotyping was performed in accordance with JAX® (the Jackson Laboratory) protocols. This strain was backcrossed to C57BL/6 for 3 generations and two more generations will be backcrossed also.

[0096] NGAL null F0 founder mice are crossed with the Cre deleter strain that expresses Cre at the one-cell stage of preimplantation embryo under the control of adenovirus EIIa promoter B6.FVB-Tg(EIIa-cre)C5379Lmgd/J (JAX® Mice Stock #003724).^{37,38} The efficiency of Cre-mediated gene rearrangement is >50% in male mice homozygous for the chromosome carrying EIIa-cre transgene X female homozygous in the immunoglobulin light chain kappa locus for loxP-neo-loxP insertional cassette. 50% of F1 showed complete

excision and the rest 50% showed partial excision of neo DNA. The complete excision was transmissible through the germ line. Genotyping was performed as per JAX® (the Jackson Laboratory) protocols. This is a congenic strain that has been backcrossed to C57BL/6 for at least 10 generations.

[0097] Neutrophil-specific Cre. There is an established Cre mouse strain which specifically expresses nuclear Cre in neutrophils and macrophages (B6.129P2-Lyzstm1(cre)Ifo/J; JAX® Mice Stock # 004781) under control of the endogenous Lysozyme M locus. This knock-in strategy for LysM-cre, rather than random transgene insertion, was especially important for this gene, since demethylation of 3' enhancer downstream of LysM gene (exon 4) is involved in myeloid specific expression.³⁹ The Cre efficiency was nearly 100% in granulocytes and 83-93% in macrophages of F1 mice double transgenic for LysM-cre X lox1P-flanked beta-polymerase gene, and 75% in neutrophils and 82-91% in macrophages for HIF-1 and VEGF conditionally null mice. The excision of loxP-flanked DNA sequences in renal cells was not examined, but, at least, overall excision frequency was very low in the lung and spleen cells. Mouse lysozyme M gene is found only at low levels in the kidney, perhaps contaminating blood (0.4% of that in mature macrophage). Genotyping was performed in accordance with JAX® (the Jackson Laboratory) protocols. The strain has been backcrossed to C57BL/6 for more than 6 generations.

[0098] In situ hybridization. NGAL RNA was detected using digoxigenin-labeled antisense riboprobes generated from cDNAs encoding NGAL (exon 1-7, 566bp) by linearization with XhoI followed by T7 RNA polymerase. Kidneys were collected in ice-cold PBS and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS), briefly quenched in 50 mM NH₄Cl, cryoprotected overnight in 30% sucrose PBS and embedded and sectioned (16µM) in Optimal Cutting Temperature (O.C.T.) compound. The sections were post-fixed in 4% paraformaldehyde (PFA) for 10 min, treated with proteinase K (1 mg/ml for 3 min), acetylated and prehybridized for 2 hrs, and then hybridized at 68-72°C overnight. The prehybridization and hybridization solution was 50% formamide, 5' SSC, 5' Denhardt's, 250 mg/ml baker's yeast RNA (Sigma), and 500 mg/ml herring sperm DNA (Sigma). Sections were washed at 72°C in 5' SSC for 5-10 minutes, then at 72°C in 0.2' SSC for 1 hour and then stained overnight (4°C) with an anti-digoxigenin antibody coupled with alkaline phosphatase (Boehringer-Mannheim), at a 1:5000 dilution in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% heat inactivated goat serum. Alkaline phosphatase activity was detected using BCIP, NBT (Boehringer-Mannheim) with 0.25 mg/ml levamisole

in a humidified chamber for 1-3 days in the dark. Sections were dehydrated and mounted in Permount (Fisher Scientific).

[0099] Western Blot. Urine and recombinant mouse NGAL protein standards were immunoblotted using polyclonal anti-NGAL antibodies (R&D Systems, Minneapolis) and donkey anti-rabbit HRP-labelled IgG antibodies (Jackson ImmunoResearch). NGAL protein was semi-quantified by comparison with standards using ImageJ software (NIH).

[00100] *In situ* hybridization and immunohistochemistry. Frozen and paraffin-embedded sections of mouse kidneys were prepared by following standard histological procedures. The paraffin sections were dewaxed and then rehydrated by using HistoClear (Fisher Scientific) and a gradient of ethanol, respectively, before *in situ* hybridization. A specific digoxigenin-labeled antisense riboprobes was generated from mouse Ngal cDNA (Genbank accession number: NM_008491) by using a Dig-labelling kit (Roche Applied Biosystems), and was hybridized and detected as previously described.⁴⁰ The hybridized sections were counterstained with methyl green, dehydrated and mounted in Permount (Fisher Scientific). Frozen and paraffin-embedded sections were used for immunohistochemical analysis. Anti-mCherry (Clontech) and anti-v-ATPase B1/2 (Santa Cruz Biotechnology) were used at a 1:50 dilution and antigen was localized by HRP-DAB chromogen (R&D Systems) staining.

[00101] Real-time PCR analysis. Total RNA was isolated with the mirVANA RNA extraction kit (Ambion), and the first strand cDNA was synthesized by using Superscript III (Invitrogen). Real-time PCR was performed to quantify Ngal mRNA expression in an iCycler MyiQ (Bio-Rad) with a SBR green supermix reagent (Bio-Rad) and Ngal-specific primers (Supplemental Table 1). β -actin was quantified as an internal control. The $\Delta\Delta CT$ method was used to calculate fold amplification of transcripts. Total RNA was isolated with the mirVANA RNA extraction kit (Ambion).

[00102] Real-Time PCR from C57BL6, Ngal^{-/-}, Myd88^{-/-}, Tlr2^{-/-}, Tlr4^{-/-} and Tlr4^{-/-} was performed according to Bio-Rad SYBR GREEN, iCyclerMyiQ protocols. Target genes, including Ngal, β -actin, utilized respectively: Ngal 116 forward primer 5'-ctcagaacttgatccctgcc-3' (SEQ ID NO.: 1) and NGALa593 reverse 5'-tccttgaggcccagacatt-3' (SEQ ID NO.: 2); β -actin 415 forward primer 5'-ctaaggccaaccgtgaaaag-3' (SEQ ID NO.: 3) and β -actin 696 reverse primer 5'-tctcagctgtggtggtgaag-3' (SEQ ID NO.: 4). The $\Delta\Delta CT$ method was used to calculate fold amplification of transcripts.

[00103] Mouse urinary tract infection. Female C57BL/6, NgaiEII-Cre, NgaiHoxB7-cre, C57B6, Tlr2-/-, Tlr4-/-, and Tlr11-/- mice were used at an age of 8-16 weeks. In short, 10-20µl of the bacterial suspension (5×10^9 colony forming units/ml) was placed into the bladder of anesthetized mice through a soft polyethylene catheter. Bacterial tissue counts were obtained after homogenization of organ and serial plating on LB plates. Urinary colony forming units (CFU) were determined by direct collection of urine from the mouse and followed by plating.

[00104] Western Blot. NGAL was quantified by western blots, using non-reducing 4-15% tris-HCL gels (Bio-Rad, Laboratories, Inc. Hercules, CA) and monoclonal (1:1000; AntibodyShop, Gentofte, Denmark) or rabbit polyclonal antibodies (R&DSystems, Minneapolis) together with standards (0.2-10 ng) of human or mouse recombinant NGAL protein. NGAL was reproducibly detected to 0.4ng/lane. NGAL expression was quantified using ImageJ software (NIMH).

References for Example 1

[00105] 1. Litwin, M.S., et al. Urologic diseases in America Project: analytical methods and principal findings. J Urol 173, 933-937 (2005).

[00106] 2. Brzuszkiewicz, E., et al. How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. Proceedings of the National Academy of Sciences of the United States of America 103, 12879-12884 (2006).

[00107] 3. McNeeley, S.G., Baselski, V.S. & Ryan, G.M. An evaluation of two rapid bacteriuria screening procedures. Obstet Gynecol 69, 550-553 (1987).

[00108] 4. Pfaller, M.A. & Koontz, F.P. Laboratory evaluation of leukocyte esterase and nitrite tests for the detection of bacteriuria. J Clin Microbiol 21, 840-842 (1985).

[00109] 5. Little, P., et al. Dipsticks and diagnostic algorithms in urinary tract infection: development and validation, randomised trial, economic analysis, observational cohort and qualitative study. Health Technol Assess 13, iii-iv, ix-xi, 1-73 (2009).

[00110] 6. Gorelick, M.H. & Shaw, K.N. Screening tests for urinary tract infection in children: A meta-analysis. Pediatrics 104, e54 (1999).

[00111] 7. Nickolas, T.L., et al. Sensitivity and specificity of a single emergency department measurement of urinary neutrophil gelatinase-associated lipocalin for diagnosing acute kidney injury. Ann Intern Med 148, 810-819 (2008).

- [00112] 8. Bennett, M., et al. Urine NGAL predicts severity of acute kidney injury after cardiac surgery: a prospective study. *Clin J Am Soc Nephrol* 3, 665-673 (2008).
- [00113] 9. Paragas, N., Qiu, A., and Barasch, J. Seeing Kidney Disease in Real-time: NGAL Reporter Mouse Detects the Response of Tubular Segments to Cell Stressors *In vivo* (In Press). *Nature medicine* (2010).
- [00114] 10. Mishra, J., et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol* 14, 2534-2543 (2003).
- [00115] 11. Mori, K., et al. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *J Clin Invest* 115, 610-621 (2005).
- [00116] 12. Mishra, J., et al. Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin. *J Am Soc Nephrol* 15, 3073-3082 (2004).
- [00117] 13. Barasch, J. & Mori, K. Cell biology: iron thievery. *Nature* 432, 811-813 (2004).
- [00118] 14. Flo, T.H., et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature* 432, 917-921 (2004).
- [00119] 15. Goetz, D.H., et al. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell* 10, 1033-1043 (2002).
- [00120] 16. Lakso, M., et al. Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. *Proceedings of the National Academy of Sciences of the United States of America* 93, 5860-5865 (1996).
- [00121] 17. Zhu, X.D. & Sadowski, P.D. Cleavage-dependent ligation by the FLP recombinase. Characterization of a mutant FLP protein with an alteration in a catalytic amino acid. *The Journal of biological chemistry* 270, 23044-23054 (1995).
- [00122] 18. Sauer, B. Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Molecular and cellular biology* 7, 2087-2096 (1987).

- [00123] 19. Yu, J., Carroll, T.J. & McMahon, A.P. Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* (Cambridge, England) 129, 5301-5312 (2002).
- [00124] 20. Bulut, Y., et al. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J Immunol* 168, 1435-1440 (2002).
- [00125] 21. Ohashi, K., Burkart, V., Flohe, S. & Kolb, H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol* 164, 558-561 (2000).
- [00126] 22. Vabulas, R.M., et al. HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J Biol Chem* 277, 15107-15112 (2002).
- [00127] 23. Okamura, Y., et al. The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem* 276, 10229-10233 (2001).
- [00128] 24. Termeer, C., et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *The Journal of experimental medicine* 195, 99-111 (2002).
- [00129] 25. Johnson, G.B., Brunn, G.J., Kodaira, Y. & Platt, J.L. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J Immunol* 168, 5233-5239 (2002).
- [00130] 26. Smiley, S.T., King, J.A. & Hancock, W.W. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* 167, 2887-2894 (2001).
- [00131] 27. El-Achkar, T.M. & Dagher, P.C. Renal Toll-like receptors: recent advances and implications for disease. *Nat Clin Pract Nephrol* 2, 568-581 (2006).
- [00132] 28. Wolfs, T.G., et al. *In vivo* expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. *J Immunol* 168, 1286-1293 (2002).
- [00133] 29. Kim, B.S., et al. Ischemia-reperfusion injury activates innate immunity in rat kidneys. *Transplantation* 79, 1370-1377 (2005).
- [00134] 30. Zhang, D., et al. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 303, 1522-1526 (2004).

- [00135] 31. Reigstad, C.S., Hultgren, S.J. & Gordon, J.I. Functional genomic studies of uropathogenic *Escherichia coli* and host urothelial cells when intracellular bacterial communities are assembled. *The Journal of biological chemistry* 282, 21259-21267 (2007).
- [00136] 32. Cirl, C., et al. Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. *Nature medicine* 14, 399-406 (2008).
- [00137] 33. Yang, J., et al. An iron delivery pathway mediated by a lipocalin. *Mol Cell* 10, 1045-1056 (2002).
- [00138] 34. Yang, J., Mori, K., Li, J.Y. & Barasch, J. Iron, lipocalin, and kidney epithelia. *Am J Physiol Renal Physiol* 285, F9-18 (2003).
- [00139] 35. Bao, G., Clifton, M., Hoette, T., Mori, K., Deng, S., Qiu, A., Viltard, M., Williams, D., Paragas, N., Leete, T., Kulkarni, R., Li, X., Lee, B., Kalandadze, A., Ratner, A., Pizarro, J., Schmidt-Ott, K., Landry, D., Raymond, K., Strong, R. and Barasch, J. Iron Traffics in Circulation Bound to Siderocalin (Ngal) Complexed with a Bacterial Metabolite Called Catechol (In Press). *Nat Chem Bio* (2010).
- [00140] 36. Rodriguez, C.I., et al. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat Genet* 25, 139-140 (2000).
- [00141] 37. Dooley, T.P., Miranda, M., Jones, N.C. & DePamphilis, M.L. Transactivation of the adenovirus EIIa promoter in the absence of adenovirus E1A protein is restricted to mouse oocytes and preimplantation embryos. *Development* 107, 945-956 (1989).
- [00142] 38. Lakso, M., et al. Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci U S A* 93, 5860-5865 (1996).
- [00143] 39. Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R. & Forster, I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 8, 265-277 (1999).
- [00144] 40. Li, J.Y., et al. Scara5 is a ferritin receptor mediating non-transferrin iron delivery. *Developmental cell* 16, 35-46 (2009).

EXAMPLE 2

[00145] The numbers in superscript below refer to the corresponding numbered reference(s) at the end of this Example.

[00146] The results presented herein, and in Example 1, demonstrate that specific segments of the kidney epithelia rapidly produce NGAL (siderocalin), which has bacteriostatic activity, blocking growth of uropathogenic bacteria located in the urinary tract, including the bladder. Thus, the kidney plays a role in innate defense. NGAL inhibits the growth of bacteria by capturing at high affinity iron bound to catecholate-siderophores and/or endogenous catechols¹, making it a potent antimicrobial by limiting *E. coli*'s access to iron. The kidney and bladder detected a urinary tract infection (UTI) in part by segmentally localized expression of Toll-like receptors (TLRs), which trigger NGAL expression. This data establishes a rationale for the abundant NGAL secretion from the kidney in both septic and perhaps in aseptic states, demonstrating that the kidney defends the urinary system via the exocrine delivery of NGAL.

[00147] In Humans^{4,5} and mice⁶ secreted lipocalin Neutrophil Gelatinase Associated Lipocalin (NGAL) is markedly upregulated and expressed by the kidney in proportion to the dose of injurious stimuli such as ischemia-reperfusion (I/R)⁶, hypoxia, drug toxicity⁶, and bacterial infection.^{4,6-9} Previously, it was not clear why this protein is expressed in the urinary system after kidney damage of different types. NGAL is a bacteriostatic protein.¹⁰ It binds catecholate-siderophore¹¹ which sequesters iron from bacteria.

[00148] In a large multi-center study it was discovered that patients with UTI caused by gram negative bacteria (n=77) had significantly elevated uNGAL compared to patients with UTI due to gram positive bacteria (n=10) (Fig. 5a, 2078±3215 vs 592±1242 mg/g creatinine, P=0.01). A dose-response relationship was seen between number of colony forming units (CFU) of UTI-causing bacteria and uNGAL levels (Fig. 5b). Patients with more than 105 CFU (n=64) had significantly more uNGAL compared to patients with between 104 and 105 CFU (n=21) (2251±3353 mg/g creatinine, 928±1850 mg/gm creatinine, P=0.02). Consistently, the number of white blood cells in the urine and uNGAL levels were also directly proportional to the amount of uNGAL. Patients with either 11-20 cells per high powered field (hpf) (n=16) or <30 cells per hpf (n=46) had significantly elevated uNGAL levels compared to patients with between 3 to 5 cells per hpf (n=10) (1455±1534 µg/g creatinine in 11-20 cells/hpf, 3023±4067 µg/g creatinine <30 cells/hpf versus 219±309 µg/g creatinine for 3-5 cells/hpf, P=0.002 and <0.001) (Fig. 5c).

[00149] To establish the relationship between NGAL and UPEC (uropathogenic *Escherichia coli*), we grew a pyelonephritogenic clinical UPEC isolate (CFT073) in M9 minimal medium supplemented with MgCl₂ and glucose (green hashed line: Fig. 1a), and

monitored bacterial growth by spectrophotometry. Bacterial growth was significantly inhibited (Fig. 1a) when the UPEC was grown with the addition of mouse (m) NGAL (red line and open red squares: 5 μ M), but it could be rescued by oversaturating NGAL with the addition of FeCl₃ (blue line with open blue circles: 1mM). This data is consistent with NGAL's activity in iron scavenging.¹¹

[00150] To determine the role NGAL has in an acute UTI *in vivo* a conditional NgalloxP/loxP animal was generated and used to generate tissue specific knockouts (Methods and). First, a NgalloxP/loxP mouse was bred with an EIIa-Cre12 mouse (NgalloxP/loxP, EIIa-Cre) which generated a knock-out of NGAL in all cells. Ngal wild-type (Ngal+/+) mice (white bar) and NgalloxP/loxP, EIIa-Cre (black bars) mice were challenged with a transurethral (TU) infection of the UPEC (10-20ul of 5x10⁸ CFU/ml CFT073) and urinary (u) NGAL and urinary colony forming units (c.f.u.) were monitored for one week (Fig. 1b). In Ngal+/+ mice we noted that the secretion of uNGAL mirrored both the onset of the urinary tract infection (UTI), indicated by log order increases in uCFU, and the resolution of the acute phase of the infection (3-4 d, Fig. 1b). uNGAL was detected within 24 h of the application of the UPEC to the bladder. Ngal+/+ mice clear the acute UTI within 3-5 d after the initial challenge of the UPEC, but animals without a functioning Ngal allele took significantly more time (6 days) than the wild type mice to clear the UPEC (NgalloxP/loxP, EIIa-Cre mice had significantly more bacteria than Ngal+/+ at 2, 3, 4 and 5 d post-TU, P< 0.05, UTest: Fig. 1b). Hence, uNGAL is necessary to rapidly clear an acute UTI by an enterochelin dependent UPEC and importantly that the protein itself is sufficient for bacteriostasis.

[00151] A striking feature of the GU infection was the distant response of the kidney to an acute bladder event. To evaluate this TU injection was performed using heat-killed CFT073 (10⁸ CFU/ml) into the mouse bladder of the NGAL bioluminescent reporter animal.⁶ TU volumes ranging from 50-200uL of bacterial detritus activated NGAL-luc2/mC expression in the bladder, ureter and the kidney. Quantitative analysis of NGAL-luc2/mC signal from the kidney revealed a 2.2 fold increase in NGAL-luc2/mC expression compared to PBS control (Fig. 6a). To determine the relative contribution of these organs to uNGAL QPCR was performed and the copy number of Ngal per organ 1 d post-TU challenge with the UPEC was quantified. Intraperitoneal (IP) injection was used as a control to stimulate Ngal expression. With either systemic or gastrourogenital application of bacteria, the kidney was the major contributor to uNGAL as measured by the number of copies of NGAL respective

of total RNA (Fig. 6b). Furthermore a two fold increase of Ngal expression was observed without the presence of bacteria in the organ. To determine the cellular source of uNGAL in response an acute UPEC infection, *in situ* hybridization was performed in Ngal^{+/+} kidneys 1 d post-TU injection of the UPEC (5 x 10⁹ CFU/ml). High levels of Ngal RNA were expressed by bladder epithelium (Fig. 6c) and through microscopic examination of the kidney tissue, Ngal mRNA was identified in epithelia of the Thick Ascending Limb of Henle (TAL) and the Collecting Ducts (CD)(Fig. 6d). Co-staining of the Ngal positive GU epithelial cells with anti-LPS antibody revealed that cells directly in contact with the UPEC were expressing Ngal. UPEC adherence to distal epithelial cells has been previously observed by Chassin et al to be specific to intercalated cells (ICs) of the collecting duct (CD).¹³ In the present experiments it was observed that a subset of these ICs, alpha-IC (A-IC), specifically recognize the bacteria and express Ngal (Fig. 6c). To evaluate whether the effect on the CD epithelia by bacteria may be a direct interaction, primary kidney cells isolated from Ngal-Luc2/mC reporter mice⁶ were used. As shown in Fig. 6a, NGAL-Luc2 expression in kidney cells was markedly upregulated (Fig. 6d) over 24 hours after an initial inoculum of bacteria. Thus, many types of GU epithelium generate Ngal RNA and the urogenital system plays an essential role in limiting the growth of UPECs via expression of the urinary bacteriostatic molecule NGAL.

[00152] Because the data showed that both the kidney and the bladder might contribute to the uNGAL pool, Ngal expression was further studied by selectively knocking out Ngal in the NGAL expressing segments of the kidney.⁶ We deleted Ngal in the collecting duct (CD) by generating a NgalloxP/loxP, HoxB7/cre14 (NgalloxP/loxP, HoxB7/cre) CKO. HoxB7/cre has been noted to be expressed in the ureteric epithelium of the distal, non-branching medullary collecting ducts and continues into the epithelium of the ureter.¹⁴ We found that the HoxB7 compartment was a major contributor to uNGAL because uNGAL protein was decreased several-fold. Moreover there was greater than a log order increase in median uCFU after a TU challenge with the UPEC compared to the wild type mice (Fig. 7a). These data support findings that Ngal expression was localized after urinary infection was localized in the collecting duct.⁶ To examine whether the bladder epithelia also contributed to uNGAL, we incubated explanted bladders from mice with UTIs and collected conditioned media. Although we detected NGAL, it was only a fraction seen in the urine over the same period (not shown). Therefore many types of GU epithelia generate uNGAL protein, but the kidney is the major contributor during a UTI. A combination of *in situ* hybridization, organ copy

number, and segment specific knockouts, indicated *in vivo* that the major source of Ngal RNA is the kidney and the major source of NGAL protein is the kidney.

[00153] Previous findings⁶ and data from isolated primary cells in Fig. 1d, revealed that bacterial gram-negative components such as lipid A bind to TLRs, such as TLR4, and activate NF- κ B⁸ which induce Ngal *in vitro*. To determine whether uNGAL originated from the kidney, we performed kidney transplantation between Tlr4-mutant CH3/HeJ mice and wild type CH3/HeOuJ mice. TLRs are receptors for bacterial infection. TLRs^{2, 4, 5, 11} have been assumed to be expressed in the GU.¹⁵ To localize these receptors *in situ* hybridization was performed to map which segments were capable of signaling via which toll-like receptor. To evaluate the contribution of TLR signaling to NGAL expression, a cross-transplant model was utilized using TLR mutants and systemic administration of LPS as a positive control. The CH3/HeJ kidneys from the LPS-insensitive mice were transplanted into CH3/HeOuJ LPS-sensitive (control) mouse bodies, and vice versa. Two weeks after graft maturation, when uNGAL and sCr had stabilized to normal values, a low dose of lipid A (1 mg/kg of body weight) was administered to induce Ngal expression in the kidney (Fig. 7b). QPCR revealed that the wild type kidney in the Tlr4-mutant body had a 15.6 ± 2.3 fold increase in Ngal expression while the Tlr4-mutant kidney in the wild type body had only a 4.5 ± 2.6 fold increase in Ngal expression. It is not surprising that there was some Ngal induction in the knockout kidney because CH3/HeJ are partial KOs, and it has also been previously shown that lipid A can signal through the MyD88-dependent pathway without a functioning Tlr4ref. These results suggests that TLR4 is the receptor to lipid A in the kidney, and indicate that NGAL is induced by LPS activation of the TLR4::NfKB pathway.

[00154] To examine the roles of TLR's in the expression of NGAL in a UTI, TU experiments were performed on C3H/OuJ and C3H HeJ TLR4 mutants to establish the signaling pathway for uNGAL expression during an acute urinary tract infection. uNGAL expression and uCFU was measured.

[00155] The results of the study described in this Example, and those described in Example 1, demonstrate that NGAL expression is stimulated by activation TLRs located in different segments of the urogenital tract, and that UTI activates NGAL in different segments. Thus, the kidney is an exocrine organ that senses the presence of UPECs via Toll-like receptors and secretes uNGAL into the urinary space to suppress the infection.

Methods

[00156] Mouse husbandry. NgallloxP/loxP, NgalEII-Cre, NgalHoxB7-cre, C57BL6, C3H/HeJ, C3H/HeOuJ, C3H/HeN, Tlr2, Tlr4, Tlr5, Tlr11, MyD88, Ticam1, and Ngal-Luc2/mC mice were raised and used in this study.

[00157] Ngal Cre-lox Targeting Construct Generation. The BAC clone was made recombinogenic by transformation with a plasmid from the Red/ET cloning kit (Gene Bridges, Heidelberg, Germany) and the homology domains were subcloned into a backbone vector by homologous recombination based Red/ET cloning method. A loxP site was inserted into intron 1, in a 2-step procedure. A loxP-flanked neo selection marker cassette (loxP-neo-loxP) is inserted by homologous recombination and then Cre is expressed in bacterial cells (EL350) to recombine the loxP sites and excise the selection marker, leaving a single loxP site. A neo cassette is inserted in a 2-step procedure into intron 5 using homologous recombination to insert a unique restriction site (Bsiw I) and then to ligate a neo cassette by conventional methods.

[00158] Electroporation into ES cells. The targeting vector was linearized, electroporated and clones selected with neo. Primers, A1, 2, 3 were 3' of the short homology arm (SA) outside the region used to generate the targeting construct and N1 was located at the 5' end of the Neo cassette amplify 2.3, 2.4, and 2.4kb fragments respectively. Control PCR used T1 and T2, which are inside the targeting construct.

[00159] Excision of neo gene F0 mice derived from ES cells are crossed with a ubiquitous FLP deleter (including germ cells) under the control of human ACTB (β actin) promoter (B6;SJLTg(ACTFLPe)9205Dym/J (JAX® Mice Stock # 003800)16.

[00160] The efficiency of FLP-excision of FRT-flanked DNA sequence was reported to 100% in F1 mice (heterozygote β actin-FLPe X heterozygote FRT-disrupted lacZ reporter gene driven by HMGC α reductase promoter/enhancer sequence). Genotyping was performed in accordance with JAX® (the Jackson Laboratory) protocols. This strain was backcrossed to C57BL/6 for 3 generations.

[00161] Ngal null F0 founder mice were crossed with the Cre deleter strain that expresses Cre at the one-cell stage of preimplantation embryo under the control of adenovirus EIIa promoter B6.FVB-Tg(EIIa-cre)C5379Lmgd/J (JAX® Mice Stock #003724).^{12,17} The efficiency of Cre-mediated gene rearrangement >50% in male mice homozygous for the chromosome carrying EIIa-cre transgene X female homozygous in the immunoglobulin light chain kappa locus for loxP-neo-loxP insertional cassette. 50% of F1 showed complete

excision and the rest 50% showed partial excision of neo DNA. The complete excision was transmissible through the germ line. Genotyping was performed in accordance with JAX® (the Jackson Laboratory) protocols. This is a congenic strain that has been backcrossed to C57BL/6 for at least 10 generations.

[00162] Neutrophil-specific Cre. There is an established Cre mouse strain which specifically expresses nuclear Cre in neutrophils and macrophages (B6.129P2-Lyzstm1(cre)Ifo/J; JAX® Mice Stock # 004781) under control of the endogenous Lysozyme M locus. This knock-in strategy for LysM-cre, rather than random transgene insertion, was especially important for this gene, since demethylation of 3' enhancer downstream of LysM gene (exon 4) is involved in myeloid specific expression.¹⁸ The Cre efficiency was nearly 100% in granulocytes and 83-93% in macrophages of F1 mice double transgenic for LysM-cre X loxP-flanked beta-polymerase gene, and 75% in neutrophils and 82-91% in macrophages for HIF-1 and VEGF conditionally null mice. The excision of loxP-flanked DNA sequences in renal cells was not examined, but, at least, overall excision frequency was very low in the lung and spleen cells. Mouse lysozyme M gene is found only at low levels in the kidney, perhaps contaminating blood (0.4% of that in mature macrophage). Genotyping was performed in accordance with JAX® (the Jackson Laboratory) protocols. The strain has been backcrossed to C57BL/6 for more than 6 generations.

[00163] In situ hybridization. NGAL RNA was detected using digoxigenin-labeled antisense riboprobes generated from cDNAs encoding Ngal (exon 1-7, 566bp) by linearization with XhoI followed by T7 RNA polymerase. Kidneys were collected in ice-cold PBS and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer saline (PBS), briefly quenched in 50 mM NH₄Cl, cryoprotected overnight in 30% sucrose PBS and embedded and sectioned (16µM) in Optimal Cutting Temperature (O.C.T.) compound. The sections were post-fixed in 4% PFA for 10 min, treated with proteinase K (1 mg/ml for 3 min), acetylated and prehybridized for 2 hrs, and then hybridized at 68-72°C overnight. The prehybridization and hybridization solution was 50% formamide, 5' SSC, 5' Denhardt's, 250 mg/ml baker's yeast RNA (Sigma), and 500 mg/ml herring sperm DNA (Sigma). Sections were washed at 72°C in 5' SSC for 5-10 minutes, then at 72°C in 0.2' SSC for 1 hour and then stained overnight (4°C) with an anti-digoxigenin antibody coupled with alkaline phosphatase (Boehringer-Mannheim), at a 1:5000 dilution in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% heat inactivated goat serum. Alkaline phosphatase activity was detected using BCIP, NBT (Boehringer-Mannheim) with 0.25 mg/ml levamisole in a

humidified chamber for 1-3 days in the dark. Sections were dehydrated and mounted in Permount (Fisher Scientific).

[00164] Bioluminescence and Fluorescence Imaging of Living Ngal-Luc2/mC Reporter Mice. Ngal-Luc2/mC reporter mice were injected ip with 150 mg/kg of D-luciferin (Caliper Life Sciences) in PBS (pH 7.0). Ten minutes later, the mice are anesthetized (2.5% isofluorane) and a whole body image was acquired for 30s using the Xenogen IVIS optical imaging system (Xenogen Corp., Alameda, CA) with an open excitation filter and an open emission filter for luminescence and fluorescence, respectively. Regions of interest (ROIs) were drawn on the dorsal side of the animal and quantified by using Living Image Software version 3.119. Counts in the ROIs were detected by a CCD camera digitizer and were converted to physical units of radiance in photons/s/cm²/steradian¹⁹.

[00165] Western Blot. Urine and recombinant mouse NGAL protein standards were immunoblotted using polyclonal anti-NGAL antibodies (R&D Systems, Minneapolis) and donkey anti-rabbit HRP-labelled IgG antibodies (Jackson ImmunoResearch). NGAL protein was semi-quantified by comparison with standards using ImageJ software (NIH).

[00166] In situ hybridization and immunohistochemistry. Frozen and paraffin-embedded sections of mouse kidneys were prepared by following standard histological procedures. The paraffin sections were dewaxed and then rehydrated by using Histoclear (Fisher Scientific) and a gradient of ethanol, respectively, before *in situ* hybridization. A specific digoxigenin-labeled antisense riboprobes was generated from mouse Ngal cDNA (Genbank accession number: NM_008491) by using a Dig-labelling kit (Roche Applied Biosystems), and was hybridized and detected as previously described²⁰. The hybridized sections were counterstained with methyl green, dehydrated and mounted in Permount (Fisher Scientific). Frozen and paraffin-embedded sections were used for immunohistochemical analysis. Anti-mCherry (Clontech) and anti-v-ATPase B1/2 (Santa Cruz Biotechnology) were used at a 1:50 dilution and antigen was localized by HRP-DAB chromogen (R&D Systems) staining.

[00167] Real-time PCR analysis. Total RNA was isolated with the mirVANA RNA extraction kit (Ambion), and the first strand cDNA was synthesized by using Superscript III (Invitrogen). Real-time PCR was performed to quantify Ngal mRNA expression in an iCycler MyiQ (Bio-Rad) with a SBR green supermix reagent (Bio-Rad) and Ngal-specific primers. β -

actin was quantified as an internal control. The $\Delta\Delta\text{CT}$ method was used to calculate fold amplification of transcripts.

[00168] Isolation and culture of primary cells. Whole kidneys were dissected from perfused Luc2/mC di-fusion reporter mice (8-12 weeks of age) and kidney cells dispersed by collagenase (2mg/ml; Sigma), followed by culture (1×10^5 / well in 24-well plates; Falcon) in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 46 mg/l L-Valine for 24 hours.

[00169] Primary cells were treated for 24 hours with 10^4 CFU/ml uropathogenic *E. coli* (CFT073) and in some cases with 100 μ g/ml gentamicin. Alternatively, primary cells were treated with Lipid A and in some cases with NF- κ B inhibitors, MG132 (Cayman Chemical), and Analogues 27, 30, and 3121 and Analogue 3022. The Luciferase substrate (Dual-GloTM Luciferase Assay System; Promega) was added and luminescence from Luc2 and fluorescence from mC (excitation of 500–550 nm and emission of 575–650 nm) were imaged in a Xenogen IVIS optical imaging system.

[00170] Total RNA was isolated with the mirVANA RNA extraction kit (Ambion).

[00171] Real-Time PCR from C57BL6, Ngal^{-/-}, Myd88^{-/-}, C3H/HeJ, CeH/HeOuJ, Tlr2^{-/-}, Tlr4^{-/-} and Tlr11^{-/-} was performed according to Bio-Rad SYBR GREEN, iCyclerMyiQ protocols. Target genes, including Ngal, β -actin, utilized respectively: Ngal 116 forward primer 5'-ctcagaacttgatccctgcc-3' SEQ ID NO.: 1) and NGALa593 reverse 5'-tccttgaggccagacactt-3' (SEQ ID NO.: 2); β -actin415 forward primer 5'-ctaaggccaaccgtgaaaag-3' (SEQ ID NO.: 3) and β -actin 696 reverse primer 5'-tctcagctgtggtggaag-3' (SEQ ID NO.: 4). The $\Delta\Delta\text{CT}$ method was used to calculate fold amplification of transcripts.

[00172] Mouse urinary tract infection. We used female C57BL/6, NgalEII-Cre, NgalHoxB7-cre, MyD88^{-/-}, C57B6, Trif, C3H/HeJ, CeH/HeOuJ, Tlr2^{-/-}, Tlr4^{-/-}, and Tlr11^{-/-} mice at an age of 8-16 weeks. In short, we placed 10-20 μ l of the bacterial suspension (5×10^9 colony forming units/ml) into the bladder of anesthetized mice through a soft polyethylene catheter. We obtained bacterial tissue counts after homogenization of organ and serial plating on LB plates. Urinary colony forming units (CFU) were determined by direct collection of urine from the mouse and followed by plating.

[00173] Human Data. This is a cross sectional analysis derived from a study of the utility of uNGAL to discriminate patients with acute kidney injury who presented to an

emergency department at three different hospital sites. All patients presenting to the ED at the three different sites who were admitted to the hospital were approached for participation in this study. A total of 2457 patients were enrolled. Patients were consented, and a sample of their urine collected. The urine was centrifuged for 10 minutes at 12,000 rpm, the supernatant collected and frozen at -80°C. Patients who were less than 18 years of age, in end-stage renal disease, had a hospital stay less than 24 hours, or already on hemodialysis were excluded. The data presented here is a cross-sectional analysis of this data to investigate relationships between uNGAL and ascending infections of the urinary tract. Patients were assigned to a group based on urinary studies and culture results done in the emergency department. Patients in this analysis were identified as having UTI, which is defined as positive urine culture of a non-contaminate organism. Patients with a UTI secondary to urinary tract obstruction were excluded as this has been shown to elevate uNGAL levels independently of UTI status (unpublished data).

[00174] SPSS version 16.0 was used for all human data analysis (SPSS, Chicago, Illinois). All continuous data were log-transformed prior to analysis and presented as non-log-transformed values. T-test for unequal variances was used for comparisons.

[00175] Stressors. Lipid A was obtained from Alexis Biochemical.

[00176] Western Blot. NGAL was quantified by western blots, using non-reducing 4-15% tris-HCL gels (Bio-Rad, Laboratories, Inc. Hercules, CA) and monoclonal (1:1000; AntibodyShop, Gentofte, Denmark) or rabbit polyclonal antibodies (R&D Systems, Minneapolis) together with standards (0.2-10 ng) of human or mouse recombinant NGAL protein. NGAL was reproducibly detected to 0.4ng/lane.

[00177] References for Example 2.

[00178] 1. Bao, G., Clifton, M., Hoette, T., Mori, K., Deng, S., Qiu, A., Viltard, M., Williams, D, Paragas, N, Leete, T, Kulkarni, R., Li, X., Lee, B., Kalandadze, A., Ratner, A., Pizarro, J., Schmidt-Ott, K., Landry, D., Raymond, K., Strong, R. and Barasch, J. Iron traffics in circulation bound to a siderocalin (Ngal)-catechol complex. Nat Chem Biol 6, 7 (2010).

[00179] 2. Litwin, M.S., et al. Urologic diseases in America Project: analytical methods and principal findings. J Urol 173, 933-937 (2005).

- [00180] 3. Brzuszkiewicz, E., et al. How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. P Natl Acad Sci USA 103, 12879-12884 (2006).
- [00181] 4. Mori, K., et al. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 115, 610-621 (2005).
- [00182] 5. Nickolas, T.L., et al. Sensitivity and specificity of a single emergency department measurement of urinary neutrophil gelatinase-associated lipocalin for diagnosing acute kidney injury. Ann Intern Med 148, 810-819 (2008).
- [00183] 6. Paragas, N., et al. The Ngal reporter mouse detects the response of the kidney to injury in real time. Nature medicine 17, 216-222 (2011).
- [00184] 7. Mishra, J., et al. Amelioration of ischemic acute renal injury by neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol 15, 3073-3082 (2004).
- [00185] 8. Mishra, J., et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. J Am Soc Nephrol 14, 2534-2543 (2003).
- [00186] 9. Barasch, J. & Mori, K. Cell biology: iron thievery. Nature 432, 811-813 (2004).
- [00187] 10. Flo, T.H., et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. Nature 432, 917-921 (2004).
- [00188] 11. Goetz, D.H., et al. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell 10, 1033-1043 (2002).
- [00189] 12. Lakso, M., et al. Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. P Natl Acad Sci USA 93, 5860-5865 (1996).
- [00190] 13. Chassin, C., et al. Renal collecting duct epithelial cells react to pyelonephritis-associated *Escherichia coli* by activating distinct TLR4-dependent and -independent inflammatory pathways. Journal of immunology (Baltimore, Md : 1950) 177, 4773-4784 (2006).

- [00191] 14. Yu, J., Carroll, T.J. & McMahon, A.P. Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development (Cambridge, England)* 129, 5301-5312 (2002).
- [00192] 15. El-Achkar, T.M. & Dagher, P.C. Renal Toll-like receptors: recent advances and implications for disease. *Nat Clin Pract Nephrol* 2, 568-581 (2006).
- [00193] 16. Rodriguez, C.I., et al. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nature Genetics* 25, 139-140 (2000).
- [00194] 17. Dooley, T.P., Miranda, M., Jones, N.C. & DePamphilis, M.L. Transactivation of the adenovirus EIIa promoter in the absence of adenovirus E1A protein is restricted to mouse oocytes and preimplantation embryos. *Development (Cambridge, England)* 107, 945-956 (1989).
- [00195] 18. Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R. & Forster, I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 8, 265-277 (1999).
- [00196] 19. Rice, B.W., Cable, M.D. & Nelson, M.B. *In vivo* imaging of light-emitting probes. *J Biomed Opt* 6, 432-440 (2001).
- [00197] 20. Li, J.Y., et al. Scara5 is a ferritin receptor mediating non-transferrin iron delivery. *Developmental cell* 16, 35-46 (2009).
- [00198] 21. Xie, Y., et al. Identification of N-(quinolin-8-yl)benzenesulfonamides as agents capable of down-regulating NFkappaB activity within two separate high-throughput screens of NFkappaB activation. *Bioorg Med Chem Lett* 18, 329-335 (2008).
- [00199] 22. Gong, G., et al. Discovery of novel small molecule cell type-specific enhancers of NF-kappaB nuclear translocation. *Bioorg Med Chem Lett* 19, 1191-1194 (2009).
- [00200] 23. Zhu, X.D. & Sadowski, P.D. Cleavage-dependent ligation by the FLP recombinase. Characterization of a mutant FLP protein with an alteration in a catalytic amino acid. *The Journal of biological chemistry* 270, 23044-23054 (1995).
- [00201] 24. Sauer, B. Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Molecular and cellular biology* 7, 2087-2096 (1987).

[00202] Although the invention has been described and illustrated in the foregoing illustrative embodiments, it is understood that the present disclosure has been made only by way of example, and that numerous changes in the details of implementation of the invention can be made without departing from the spirit and scope of the invention, which is limited only by the claims that follow. Features of the disclosed embodiments can be combined and rearranged in various ways within the scope and spirit of the invention.

CLAIMS

What is claimed is:

1. A method for treating or preventing a bacterial infection of the urinary tract, or urosepsis associated therewith, in a subject, the method comprising administering to the subject a therapeutically effective amount of one or more agents selected from the group consisting of: (a) an agent that stimulates genito-urinary tract epithelial cells to produce NGAL, (b) an NGAL protein, and (c) a functional derivative of an NGAL protein.
2. The method of claim 1, wherein the subject is a human.
3. The method of claim 1, wherein the bacterial infection is an infection with a catecholate siderophore-dependent bacterium.
4. The method of claim 1, wherein the the bacterial infection is an infection with an enterochelin-dependent bacterium.
5. The method of claim 1, wherein the the bacterial infection is an infection with an enterochelin-dependent *E. coli* bacterium.
6. The method of claim 1, further comprising administering to the subject an additional bacteriostatic or antibiotic agent.
7. The method of claim 1, wherein the genito-urinary tract epithelial cells are kidney epithelial cells.
8. The method of claim 7, wherein the kidney epithelial cells are collecting duct epithelial cells or epithelial cells of the thick ascending limb of Henlé.
9. The method of claim 1, wherein the agent that stimulates genito-urinary tract epithelial cells to produce NGAL is an NFκB activator, a NRF2 modulator, or a HIF modulator.

10. The method of claim 1, wherein the agent that stimulates genito-urinary tract epithelial cells to produce NGAL is an activator of a TLR-NFκB pathway.
11. The method of claim 10, wherein the activator of a TLR-NFκB pathway is an activator of a TLR4-NFκB pathway.
12. The method of claim 10, wherein the activator of a TLR-NFκB pathway is an activator of a TLR11-NFκB pathway.
13. The method of claim 1, wherein the agent that stimulates genito-urinary tract epithelial cells to produce NGAL is a non-toxic derivative of either lipid A, lipopolysaccharide, or endotoxin.
14. The method of claim 1, wherein the agent that stimulates genito-urinary tract epithelial cells to produce NGAL, and/or the NGAL or functional derivative thereof, are administered systemically.
15. The method of claim 1, wherein the agent that stimulates genito-urinary tract epithelial cells to produce NGAL, and/or the NGAL or functional derivative thereof, are administered locally to the genitourinary tract.
16. A method for treating or preventing bacterial infection of the urinary tract, or urosepsis associated therewith, in a subject, the method comprising stimulating genito-urinary tract epithelial cells of the subject to produce NGAL.
17. The method of claim 16, wherein the subject is a human.
18. The method of claim 16, wherein the bacterial infection is an infection with a catecholate siderophore-dependent bacterium.
19. The method of claim 16, wherein the the bacterial infection is an infection with an enterochelin-dependent bacterium.
20. The method of claim 16, wherein the the bacterial infection is an infection with an enterochelin-dependent *E. coli* bacterium.

21. The method of claim 16, wherein the genito-urinary tract epithelial cells are kidney epithelial cells.
22. The method of claim 21, wherein the kidney epithelial cells are collecting duct epithelial cells or epithelial cells of the thick ascending limb of Henlé.
23. The method of claim 16, wherein the step of stimulating genito-urinary tract epithelial cells to produce NGAL comprises administering to the subject a therapeutically effective amount one or more agent selected from the group consisting of: (a) a therapeutically effective derivative of lipid A, (b) a therapeutically effective derivative of lipopolysaccharide, (c) a therapeutically effective derivative of endotoxin, (d) an activator of the TLR4-NFκB pathway, (e) an activator of the TLR11-NFκB pathway, (f) an NFκB activator, (g) a NRF2 modulator, and (h) a HIF modulator.
24. The method of claim 23, wherein the agent is administered systemically.
25. The method of claim 23, wherein the agent is administered locally to the genitourinary tract.
26. A pharmaceutical composition for use in treating or preventing a bacterial urinary tract infection, or urosepsis associated therewith, the composition comprising a therapeutically effective amount of an agent that stimulates genito-urinary tract epithelial cells to produce NGAL, and a therapeutically effective amount of an NGAL protein or a functional derivative thereof.
27. The composition of claim 26, wherein the agent that stimulates genito-urinary tract epithelial cells to produce NGAL is selected from the group consisting of: (a) a non-toxic derivative of lipid A, (b) a non-toxic derivative of lipopolysaccharide, (c) a non-toxic derivative of endotoxin, (d) an activator of the TLR4-NFκB pathway, (e) an activator of the TLR11-NFκB pathway, (f) an NFκB activator, (g) a NRF2 modulator, and (h) a HIF modulator.

28. A method of identifying an agent that stimulates epithelial cells of the urinary tract to produce NGAL mRNA or NGAL protein, the method comprising:
- (a) providing a test population of urinary tract epithelial cells and a control population of urinary tract epithelial cells,
 - (b) contacting the test population of urinary tract epithelial cells with one or more test agents,
 - (c) contacting the control population of urinary tract epithelial cells with no agent or with one or more negative control agents, and
 - (d) determining the level of NGAL mRNA or NGAL protein in the test population and the control population, or in a culture supernatant thereof,
- wherein a level of NGAL mRNA or NGAL protein in the test population, or a culture supernatant thereof, that is higher than the level of NGAL mRNA or NGAL protein in the control population, or a culture supernatant thereof, indicates that the test agent is an agent that stimulates production of NGAL mRNA or NGAL protein by the urinary tract epithelial cells.
29. A method of identifying an agent that stimulates epithelial cells of the urinary tract to produce NGAL mRNA or NGAL protein, the method comprising:
- (a) providing a population of urinary tract epithelial cells,
 - (b) determining the control level of NGAL mRNA or NGAL protein in the population of urinary tract epithelial cells, or in a culture supernatant thereof, wherein the control level is the level of NGAL mRNA or NGAL protein present prior to contacting the urinary tract epithelial cells with one or more test agents,
 - (c) contacting the urinary tract epithelial cells with one or more test agents,
 - (d) determining the test level of NGAL mRNA or NGAL protein in the population of urinary tract epithelial cells, or in a culture supernatant thereof, wherein the test level is the level of NGAL mRNA or NGAL protein present subsequent to contacting the urinary tract epithelial cells with the one or more test agents,
- wherein if the test level of NGAL mRNA or NGAL protein exceeds the control level of NGAL mRNA or NGAL protein, the test agent is an agent that stimulates production of NGAL mRNA or NGAL protein by the urinary tract epithelial cells.

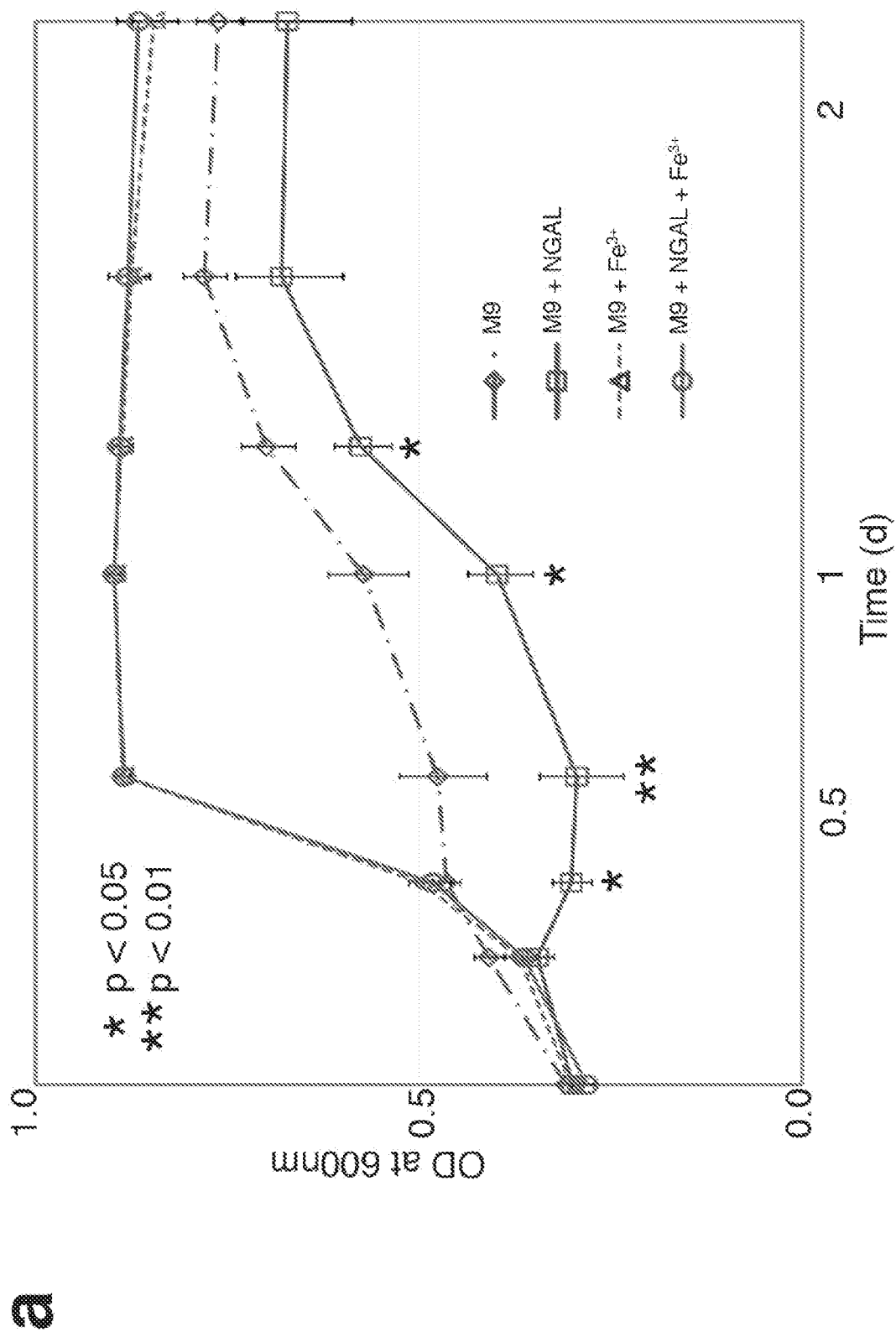


Figure 1

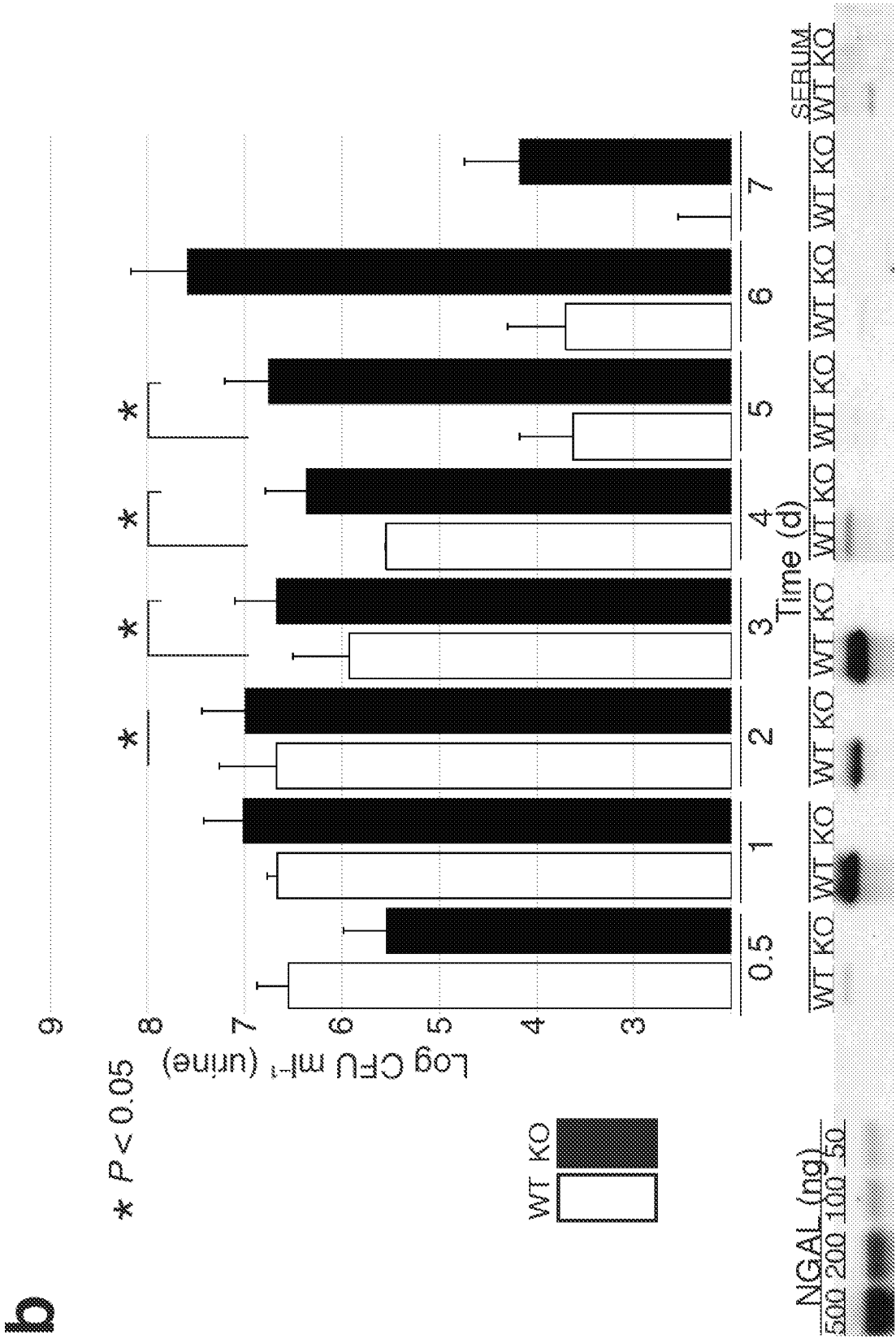


Figure 1

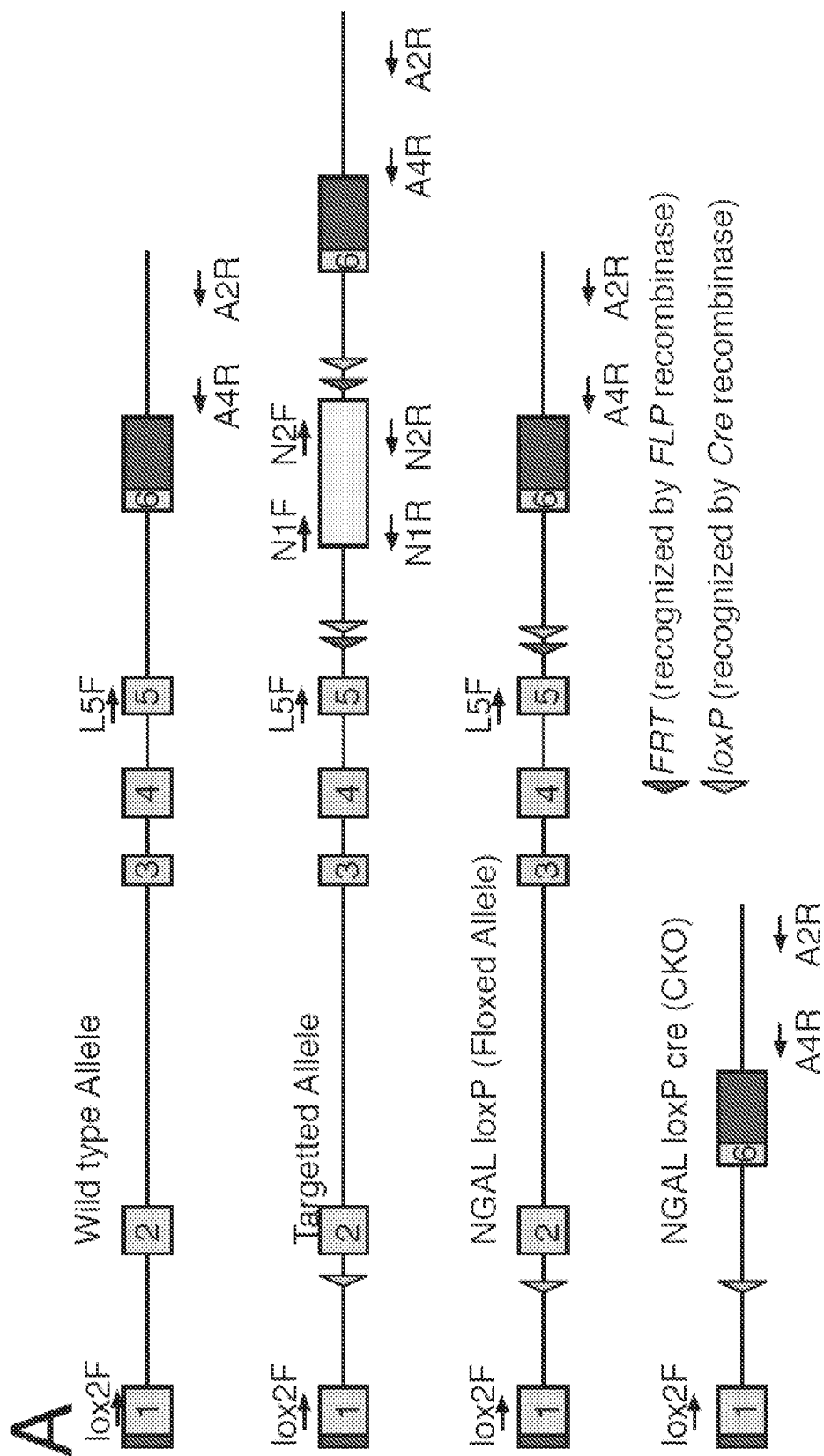
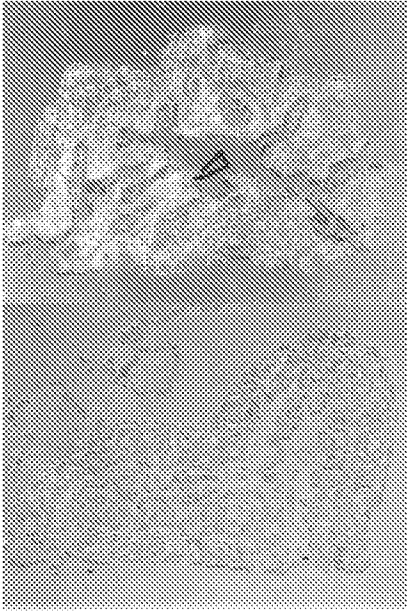


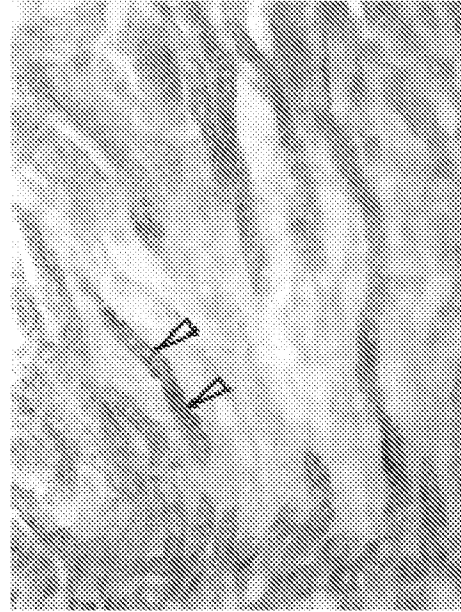
Figure 2

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a



b



c

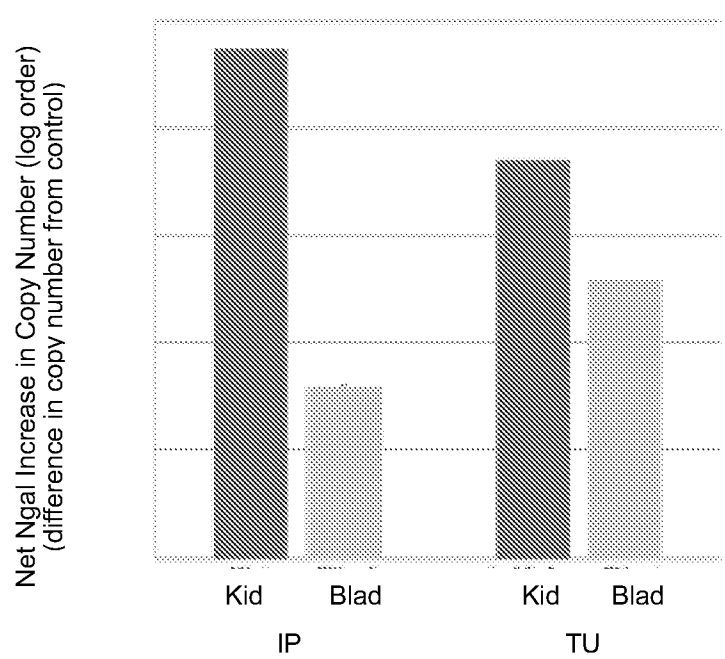


Figure 3

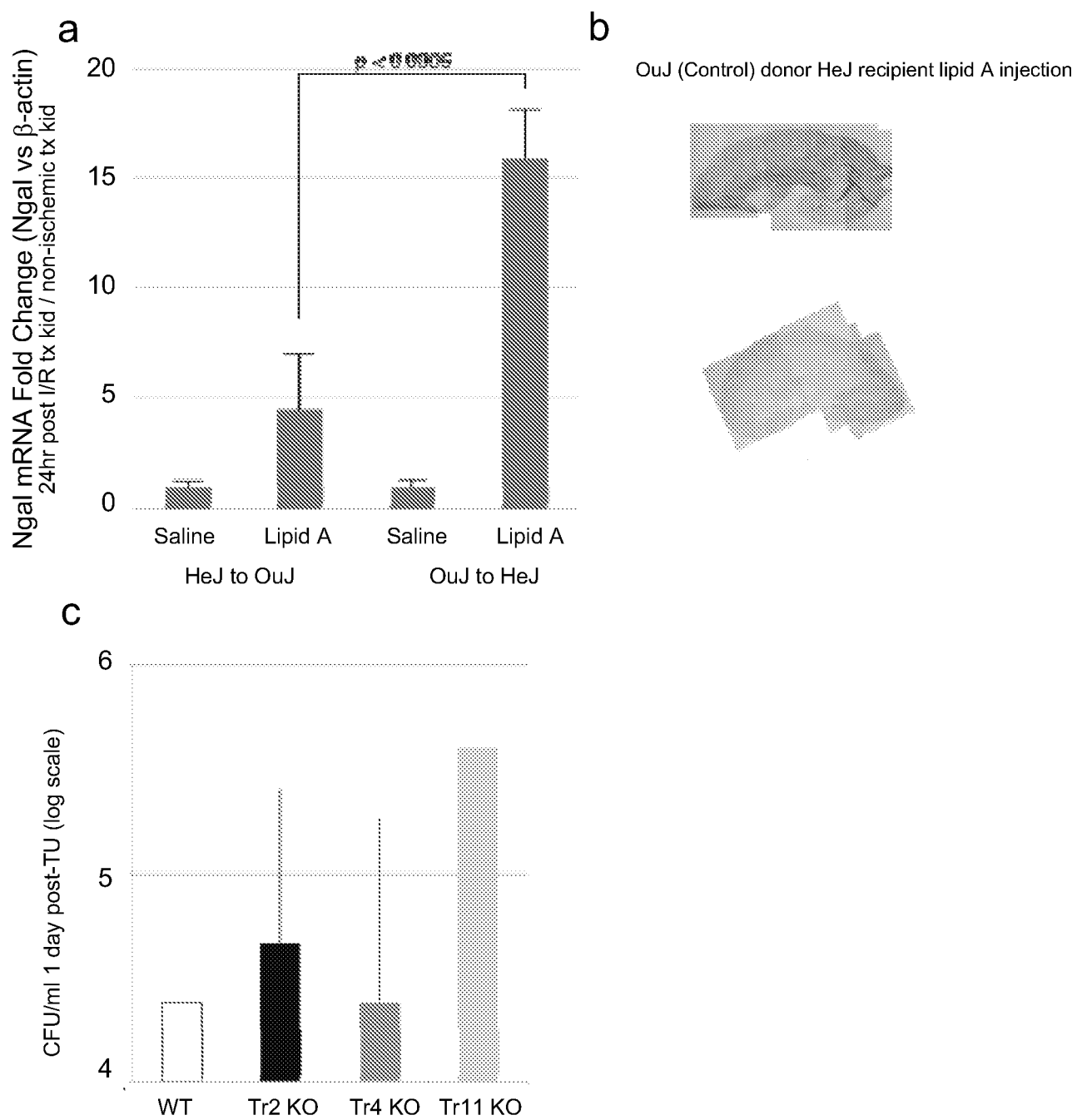


Figure 4

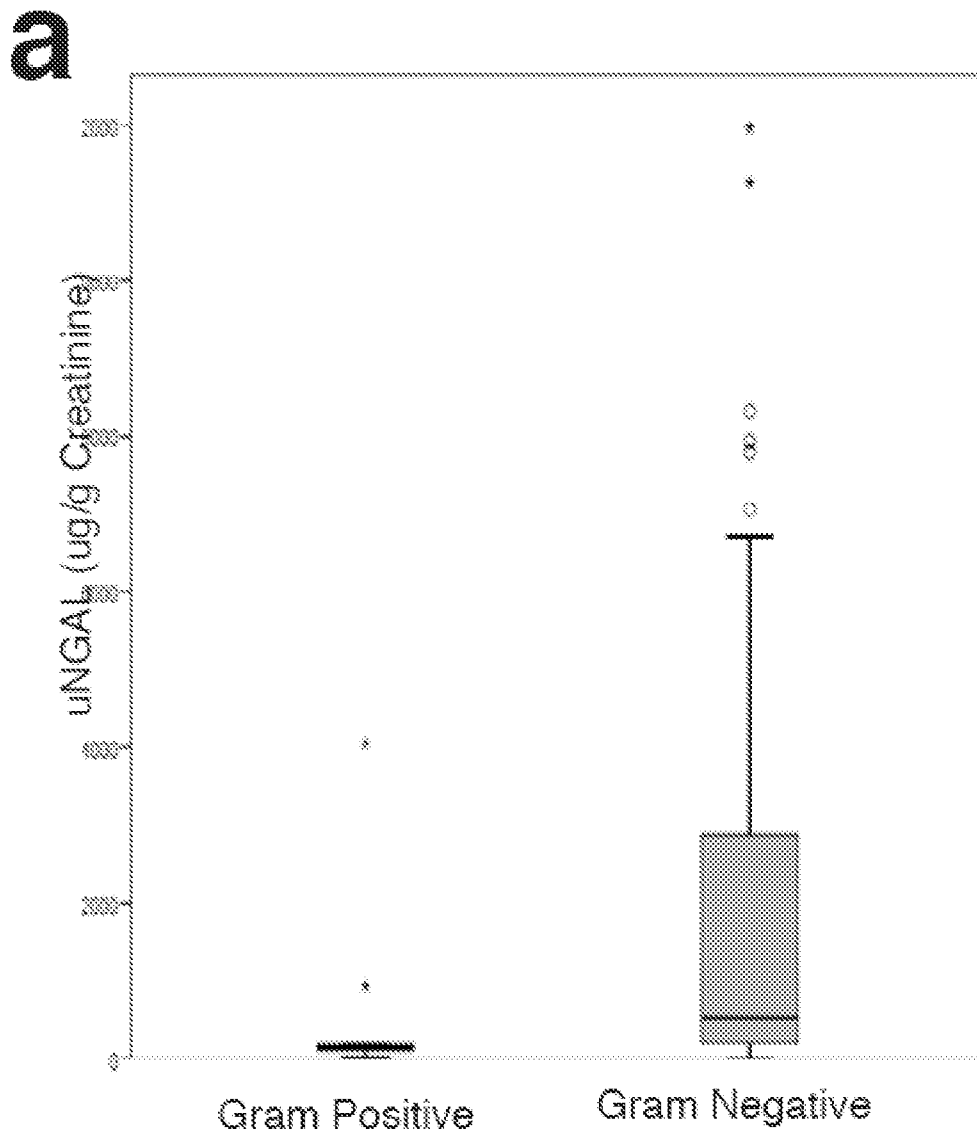


Figure 5A

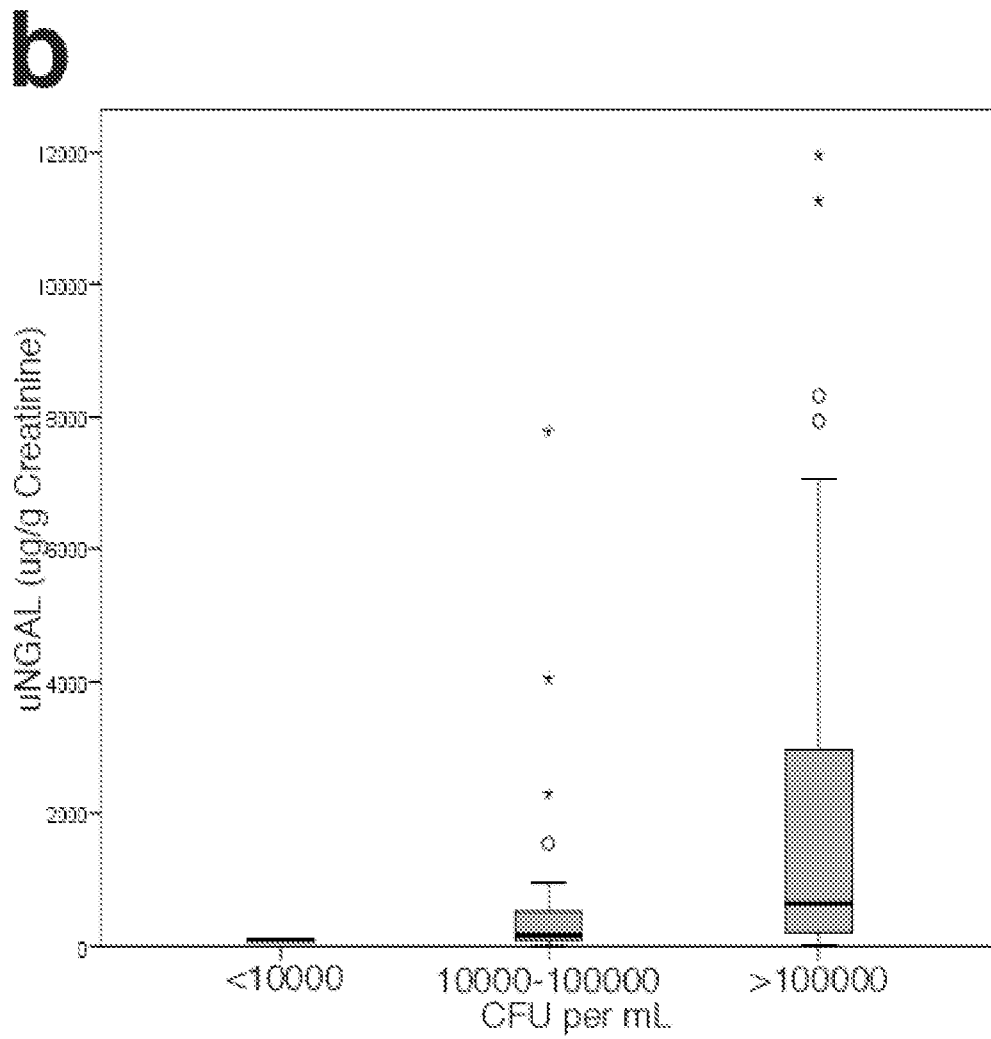


Figure 5B

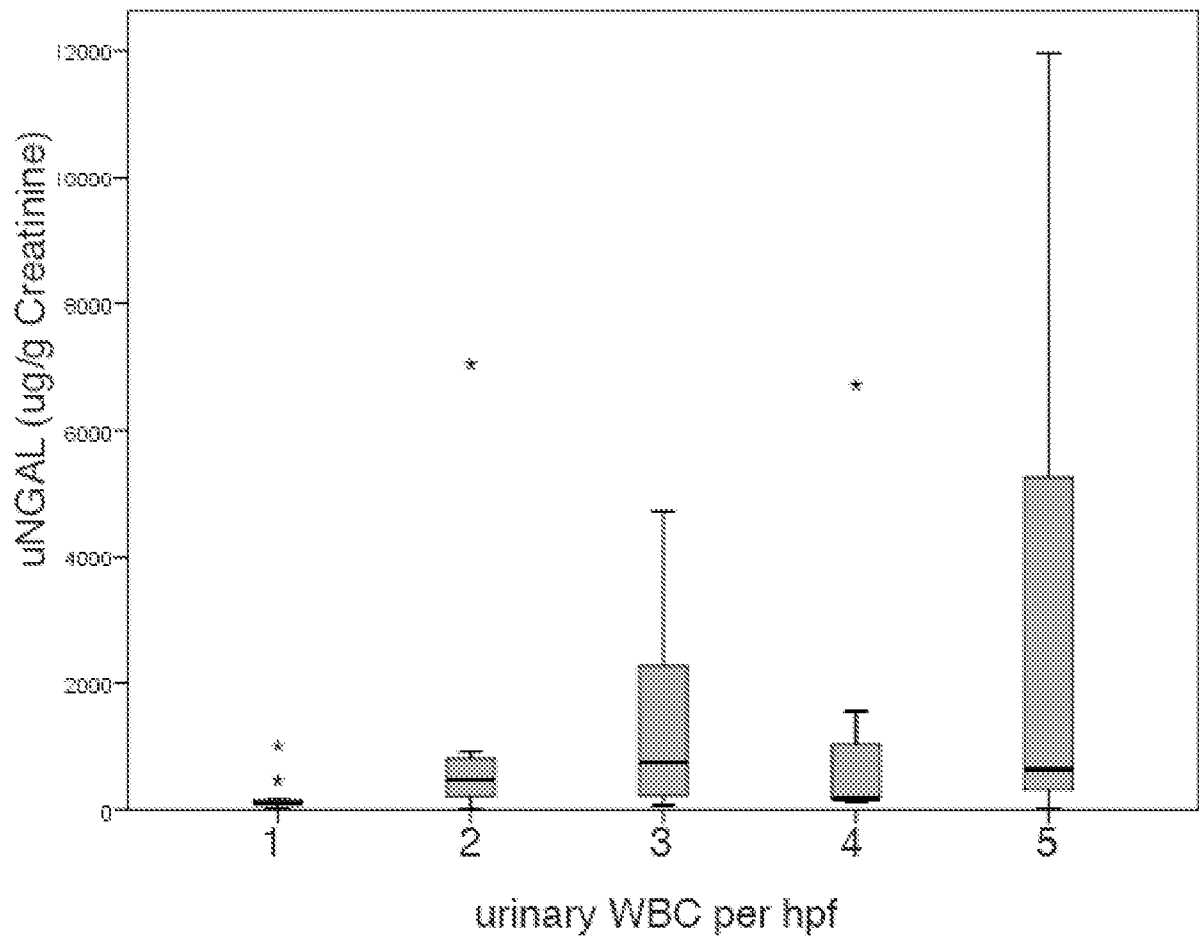
C

Figure 5C

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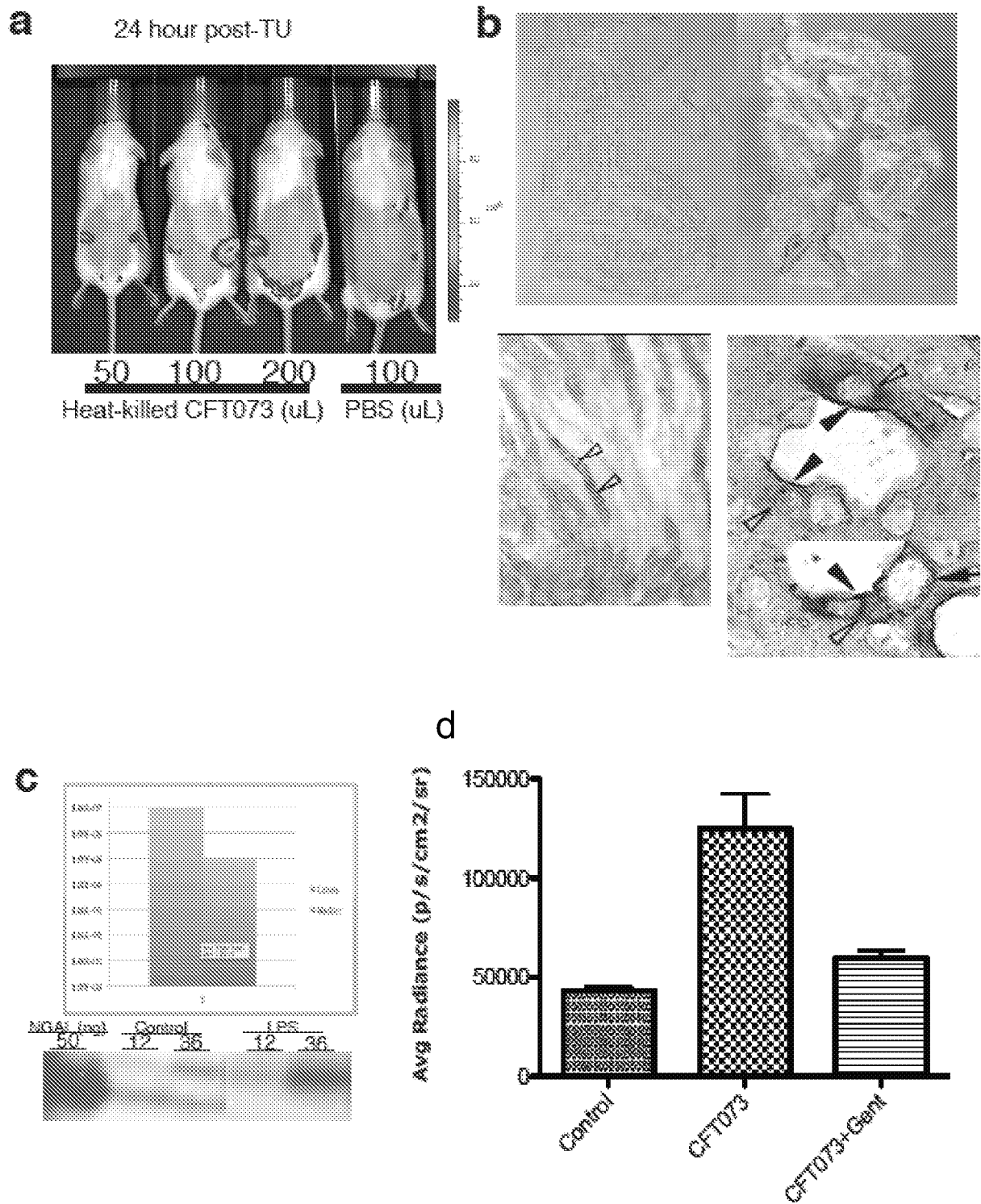


Figure 6

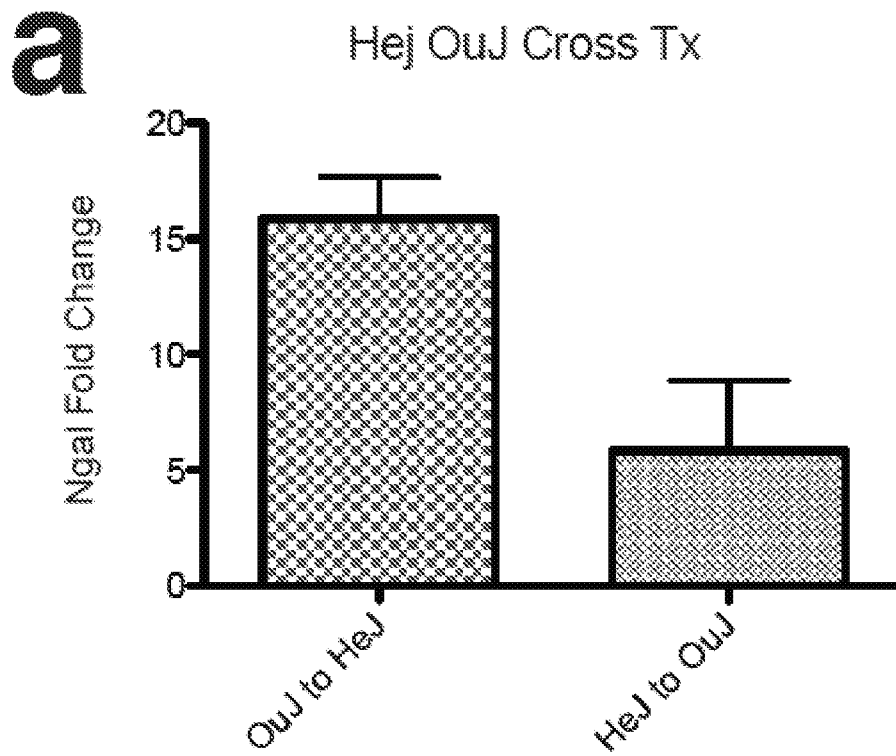


Figure 7A

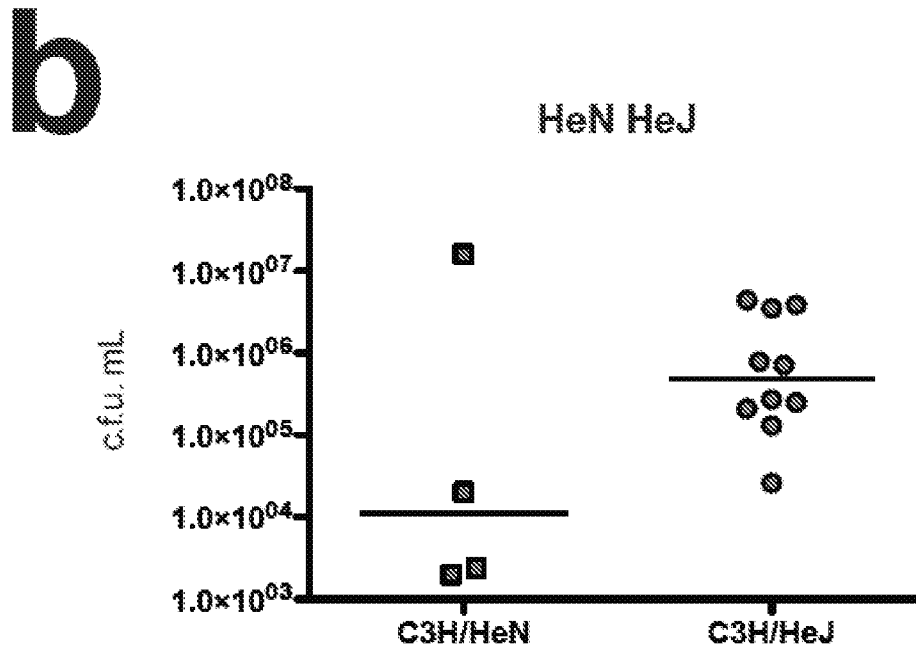


Figure 7B

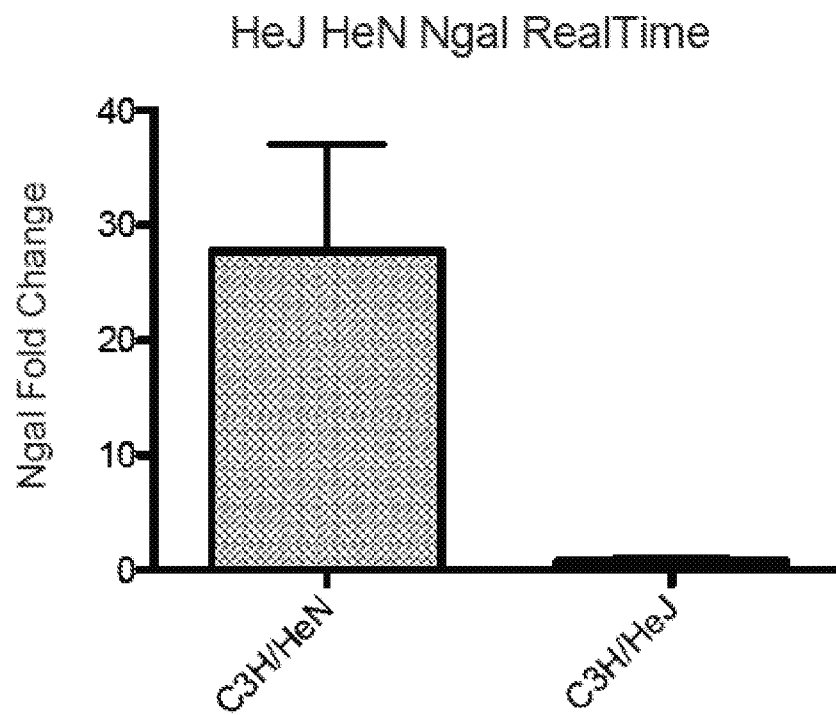
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Figure 7C

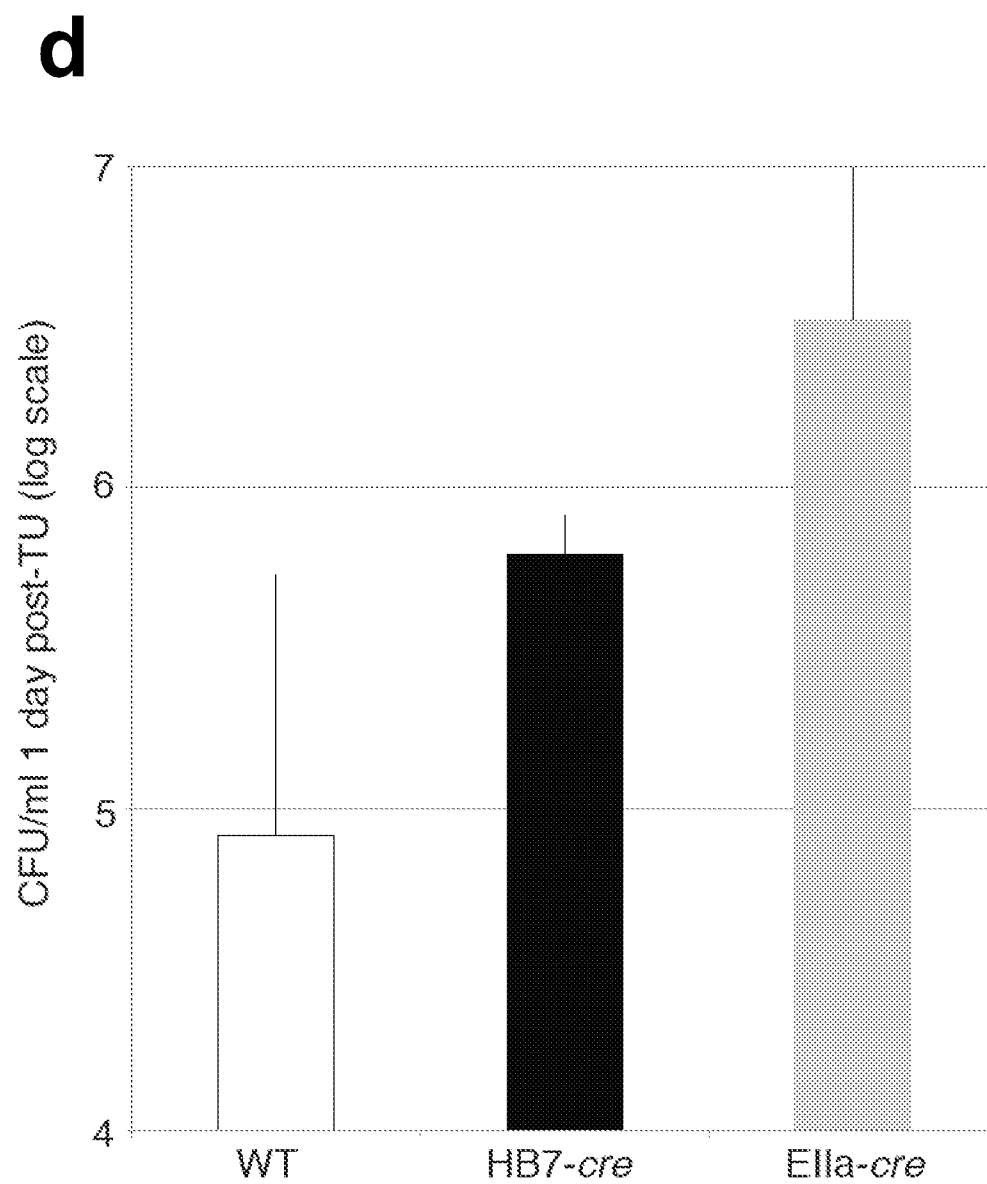


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/35757

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/07, 5/16 (2011.01)

USPC - 435/332

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 435/332

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/405; 435/7.23, 435/7.92, 435/18, 435/330, 435/333, 435/334, 435/335, 435/336, 435/340, 435/342, 435/343, 435/344; 436/63, 436/64, 436/86, 436/501; 600/573 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST (PGPB,USPT,EPAB,JPAB): NGAL, urinary tract, epithelial, infection, siderophore, catecholate, enterochelin, enterobactin, HIF, NRF2, NFkB, stimulate, produce
— Please see extra sheet for continuation —

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 2005/0261191 A1 (BARASCH et al.) 24 November 2005 (24.11.2005) abstract; para [0018], [0023], [0031]-[0032], [0096], [0101], [0115], [0118]-[0119], [0140], [0177].	1-2, 6-7 ----- 3-5, 8-27
X — Y	GWIRA et al. Expression of Neutrophil Gelatinase-associated Lipocalin Regulates Epithelial Morphogenesis in Vitro. J of Biol Chem., 2005, Vol 280, pp 7875-7882; especially pg 7875, abstract; pg 7875, right col, second para; pg 7876, left col, second and third para; 7877, left col, third para; pg 7877, Fig 1; pg 7881, Fig 6	28 ----- 9-27, 29
Y	GOETZ et al. The Neutrophil Lipocalin NGAL Is a Bacteriostatic Agent that Interferes with Siderophore-Mediated Iron Acquisition. Molecular Cell., 2002, Vol 10, pp 1033-1043; especially pg 1033, abstract; pg 1035, right col, second para; pg 1040, left col, second para	3-5, 18-20
Y	US 2008/0014644 A1 (BARASCH et al.) 17 January 2008 (17.01.2008) abstract; para [0065]	8, 22
Y	MATSUO et al. Crucial roles of binding sites for NF-kappaB and C/EBPs in IkappaB-gamma - mediated transcriptional activation. Biochem. J., 2007, Vol 405, pp 605-615, especially pg 605, abstract; pg 609, right col, second para; pg 614, left col, fourth para	9-13, 23-25, 27

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

11 July 2011 (11.07.2011)

Date of mailing of the international search report

27 JUL 2011

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/35757

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/030790 A2 (HOOK et al.) 18 March 2010 (18.03.2010) para [0015], [0144]-[00145], [00149]	12
Y	US 2007/0196876 A1 (MOSES et al.) 23 August 2007 (23.08.2007) para [0009], [0039]	29

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/35757

Continuation of (B): Fields Searched - Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Google Scholar: NGAL catecholate siderophore enterochelin e.coli screen stimulate epithelial cells urinary tract lipopolysaccharide

Google Web: TLR-NFkB NGAL stimulate the epithelial cells of urinary tract to produce NGAL production NGAL

esp@cenet: NGAL, urinary, Columbia, Barasch