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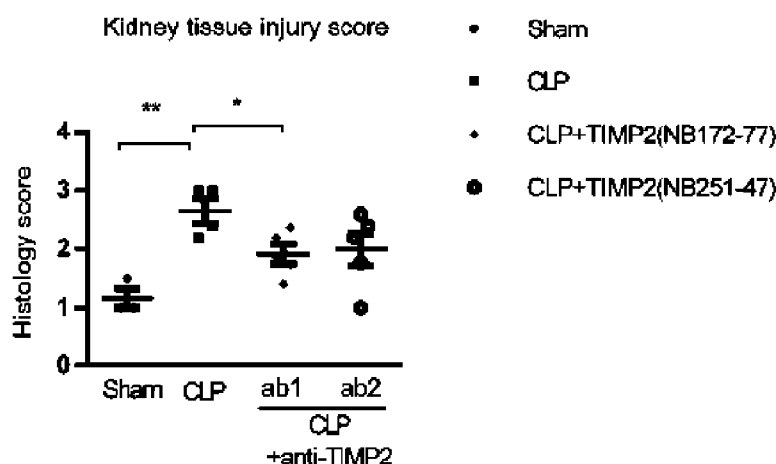
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(54) Title: USE OF ANTIBODIES TO TIMP-2 FOR THE IMPROVEMENT OF RENAL FUNCTION

FIG. 1



(57) Abstract: The invention provides methods for the treatment of subjects having or at risk of having kidney injuries using antibodies that specifically bind Metalloproteinase inhibitor 2 (TIMP-2). Specifically, the methods can be used for the treatment of a subject having chronic kidney disease (CKD), acute kidney injury (AKI), or a subject having an existing diagnosis of one or more of congestive heart failure, preeclampsia, eclampsia, diabetes mellitus, hypertension, coronary artery disease, proteinuria, glomerular filtration below the normal range, cirrhosis, serum creatinine above the normal range, sepsis, or acute renal failure (ARF).



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**USE OF ANTIBODIES TO TIMP-2 FOR THE IMPROVEMENT OF RENAL
FUNCTION**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Patent Application 62/414,479 filed October 28, 2016, which is hereby incorporated by reference in its entirety including all tables, figures and claims.

BACKGROUND

[0002] The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0003] Metalloproteinase inhibitor 2 (human precursor Swiss-Prot P16035, also known as “Tissue inhibitor of metalloproteinases 2” and “TIMP2”) is a secreted protein which complexes with metalloproteinases and irreversibly inactivates them by binding to their catalytic zinc cofactor. TIMP2 is known to act on MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-13, MMP-14, MMP-15, MMP-16 and MMP-19. TIMP2 reportedly suppresses the proliferation of endothelial cells. As a result, the encoded protein has been suggested to have a role in the maintenance of tissue homeostasis by suppressing the proliferation of quiescent tissues in response to angiogenic factors, and by inhibiting protease activity in tissues undergoing remodeling.

[0004] In addition, WO2010/048346 and WO2011/075744, each of which is hereby incorporated by reference in its entirety including all tables, figures and claims, describe the use of TIMP2 for evaluating the renal status of a subject both individually and in multimarker panels. In particular, TIMP2 levels measured by immunoassay are shown to correlate to risk stratification, diagnosis, staging, prognosis, classifying and monitoring of renal status.

[0005] Acute kidney injury (AKI) is due to a variety of conditions and has serious consequences. AKI is defined as any of the following:

Increase in SCr by ≥ 0.3 mg/dl (≥ 26.5 μ mol/l) within 48 hours; or

Increase in SCr to ≥ 1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or

Urine volume <0.5 ml/kg/h for 6 hours.

[0006] AKI is staged for severity according to the following criteria:

Stage	Serum creatinine	Urine output
1	1.5–1.9 times baseline; or ≥ 0.3 mg/dl (≥ 26.5 mmol/l) increase	<0.5 ml/kg/h for 6–12 hours
2	2.0–2.9 times baseline	<0.5 ml/kg/h for ≥ 12 hours
3	3.0 times baseline; or initiation of renal replacement therapy; or Increase in serum creatinine to ≥ 4.0 mg/dl (≥ 353.6 mmol/l); or in patients <18 years, decrease in eGFR to <35 ml/min per 1.73 m ²	<0.5 ml/kg/h for ≥ 12 hours; or anuria for ≥ 12 hours

[0007] Importantly, by defining the syndrome of acute changes in renal function more broadly, RIFLE criteria move beyond ARF. The concept of AKI, as defined by RIFLE criteria, includes other, less severe conditions. Bellomo *et al.*, *Crit Care*. 8(4):R204–12, 2004, describes the RIFLE criteria:

“Risk”: serum creatinine increased 1.5 fold from baseline OR urine production of <0.5 ml/kg body weight/hr for 6 hours;

“Injury”: serum creatinine increased 2.0 fold from baseline OR urine production <0.5 ml/kg/hr for 12 h;

“Failure”: serum creatinine increased 3.0 fold from baseline OR creatinine >355 μ mol/l (with a rise of >44) or urine output below 0.3 ml/kg/hr for 24 h or anuria for at least 12 hours;

“Loss”: persistent need for renal replacement therapy for more than four weeks.

“ESRD”: end stage renal disease—the need for dialysis for more than 3 months.

[0008] As discussed in Kellum, *Crit. Care Med.* 36: S141-45, 2008 and Ricci *et al.*, *Kidney Int.* 73, 538-546, 2008, each hereby incorporated by reference in its entirety, the RIFLE criteria provide a uniform definition of AKI which has been validated in numerous studies.

[0009] It is widely recognized that AKI leads to high morbidity and mortality in hospitalized patients, and there is an urgent need for effective therapy. Except for a few isolated studies, the vast majority of animal and clinical studies have yet to demonstrate

conclusively the benefit of a pharmacologic treatment of AKI. Jo et al., Clin J Am Soc Nephrol 2: 356–365, 2007.

SUMMARY

[0010] It is an object of the invention to methods for the treatment of subjects having, or at risk of, renal injuries. Specifically, the administration of antibodies directed to Metalloproteinase inhibitor 2 (TIMP-2) are shown herein to improve kidney histology scores in a model of sepsis-induced AKI.

[0011] In a first aspect, the present invention provides methods for improving kidney function in a subject, most preferably a human subject, in need thereof comprising: administering to the subject an antibody that specifically binds Metalloproteinase inhibitor 2 (TIMP-2).

[0012] In certain embodiments, the administration of the anti-TIMP-2 antibody is performed optionally in association with one or more further therapeutic agents or therapeutic procedures indicated for the improvement of kidney function, in an amount sufficient to improve kidney function. Suitable additional treatment modalities are described in detail hereinafter.

[0013] In certain embodiments a subject in need thereof is a subject having chronic kidney disease (CKD) or that exhibits one or more symptoms of CKD. In other embodiments, a subject in need thereof is a subject having acute kidney injury (AKI) or that exhibits one or more symptoms of AKI.

[0014] In still other embodiments, a subject in need thereof is a subject identified as being at increased risk of AKI. By way of example, such a subject may be identified as being at increased risk based on an existing diagnosis of one or more of congestive heart failure, preeclampsia, eclampsia, diabetes mellitus, hypertension, coronary artery disease, proteinuria, renal insufficiency, glomerular filtration below the normal range, cirrhosis, serum creatinine above the normal range, sepsis, injury to renal function, or reduced renal function. In certain embodiments, the subject is characterized as having diabetic nephropathy (DN) or exhibiting one or more symptoms of DN.

[0015] Alternatively, or in addition, such a subject may be identified as being at increased risk based on the subject undergoing or having undergone major vascular surgery, coronary artery bypass, or other cardiac surgery, and/or has received one or more of NSAIDs, cyclosporines, tacrolimus, aminoglycosides, foscarnet, ethylene glycol,

hemoglobin, myoglobin, ifosfamide, heavy metals, methotrexate, radiopaque contrast agents, or streptozotocin.

[0016] In certain embodiments such a subject may be identified as being at increased risk based on a biomarker result. Preferably, the biomarker result comprises one or more of a measured urinary TIMP-2 concentration and a measured urinary Insulin-like growth factor-binding protein 7 (IGFBP7) concentration, and most preferably a combined result calculated from a measured urinary TIMP-2 concentration and a measured urinary IGFBP7 concentration, such as a [TIMP-2]_x[IGFBP7] result.

[0017] In certain embodiments, the subject is characterized as at or below AKIN stage 1; the subject is characterized as at or below AKIN stage 2, or the subject is characterized as at or below AKIN stage 3.

[0018] In various embodiments, administration of the anti-TIMP-2 antibody, alone or with the optional treatment modalities, results in an improvement in estimated glomerular filtration rate (eGFR) of the subject.

[0019] In various embodiments, administration of the anti-TIMP-2 antibody, alone or with the optional treatment modalities, reduces the level of serum creatinine in the subject.

[0020] In various embodiments, administration of the anti-TIMP-2 antibody is parenteral, such as intravenous, intraarterial, or subcutaneous.

[0021] In various embodiments, administration of the anti-TIMP-2 antibody is an IgG, an Fab fragment, an F(ab')₂, or an scFv. This list is not meant to be limiting. In certain embodiments, the anti-TIMP-2 antibody is humanized or fully human.

[0022] In certain embodiments, the one or more further therapeutic agents or therapeutic procedures indicated for the improvement of kidney function comprise one or more treatments selected from the group consisting of renal replacement therapy, management of fluid overload, administration of a caspase inhibitor, administration of minocycline, administration of a Poly ADP-ribose polymerase inhibitor, administration of an iron chelator, administration of a treatment for sepsis in a subject in need thereof, administration of insulin, administration of erythropoietin, and administration of a vasodilator.

[0023] The details of one or more embodiments of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the disclosure will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description serve to explain the principles of the invention.

[0025] FIG. 1 shows kidney tissue injury as measured by histology in a mouse cecal ligation and puncture sepsis model following treatment with anti-TIMP2 as compared to untreated sepsis animals.

[0026] FIG. 2 shows kidney function as measured by serum creatinine in a mouse cecal ligation and puncture sepsis model following treatment with anti-TIMP2 as compared to untreated sepsis animals.

DETAILED DESCRIPTION

[0027] Definitions

[0028] As used herein, the terms “Metalloproteinase inhibitor 2” and “TIMP-2” refer to one or more polypeptides present in a biological sample that are derived from the Metalloproteinase inhibitor 2 precursor (human precursor: Swiss-Prot P16035 (SEQ ID NO: 10)).

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      10      20      30      40      50      60
MGAAARTLRL ALGLLLLATL LRPADACSCS PVHPQQAFCN ADVVIRAKAV SEKEVDSGND

      70      80      90     100     110     120
IYGNPIKRIQ YEIKQIKMFK GPEKDIEFIY TAPSSAVCGV SLDVGGKKEY LIAGKAEGDG

     130     140     150     160     170     180
KMHITLCDFI VPWDTLSTTQ KKS LNHR YQM GCECKITRCP MIPCYISSPD ECLWMDWVTE

     190     200     210     220
KNINGHQAKF FACIKRSDGS CAWYRGAAPP KQEFLDIEDP

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[0029] The following domains have been identified in Metalloproteinase inhibitor 2:

Residues	Length	Domain ID
1-26	26	Signal peptide

27-220 194

Metalloproteinase inhibitor 2

[0030] Unless specifically noted otherwise herein, the definitions of the terms used are standard definitions used in the art of pharmaceutical sciences. As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

[0031] The term "subject" as used herein refers to a human or non-human organism. Thus, the methods and compositions described herein are applicable to both human and veterinary disease. Further, while a subject is preferably a living organism, the invention described herein may be used in post-mortem analysis as well. Preferred subjects are humans, and most preferably "patients," which as used herein refers to living humans that are receiving medical care for a disease or condition. This includes persons with no defined illness who are being investigated for signs of pathology.

[0032] The term "diagnosis" as used herein refers to methods by which the skilled artisan can estimate and/or determine the probability ("a likelihood") of whether or not a patient is suffering from a given disease or condition. In the case of the present invention, "diagnosis" includes using the results of an assay, most preferably an immunoassay, for a kidney injury marker of the present invention, optionally together with other clinical characteristics, to arrive at a diagnosis (that is, the occurrence or nonoccurrence) of an acute renal injury or ARF for the subject from which a sample was obtained and assayed. That such a diagnosis is "determined" is not meant to imply that the diagnosis is 100% accurate. Many biomarkers are indicative of multiple conditions. The skilled clinician does not use biomarker results in an informational vacuum, but rather test results are used together with other clinical indicia to arrive at a diagnosis. Thus, a measured biomarker level on one side of a predetermined diagnostic threshold indicates a greater likelihood of the occurrence of disease in the subject relative to a measured level on the other side of the predetermined diagnostic threshold.

[0033] Similarly, a prognostic risk signals a probability ("a likelihood") that a given course or outcome will occur. A level or a change in level of a prognostic indicator, which in turn is associated with an increased probability of morbidity (e.g., worsening renal function, future ARF, or death) is referred to as being "indicative of an increased likelihood" of an adverse outcome in a patient.

[0034] The term “antibody” as used herein refers to a peptide or polypeptide derived from, modeled after or substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, capable of specifically binding an antigen or epitope. See, e.g. Fundamental Immunology, 3rd Edition, W.E. Paul, ed., Raven Press, N.Y. (1993); Wilson (1994; J. Immunol. Methods 175:267-273; Yarmush (1992) J. Biochem. Biophys. Methods 25:85-97. The term antibody includes antigen-binding portions, i.e., “antigen binding sites,” (e.g., fragments, subsequences, complementarity determining regions (CDRs)) that retain capacity to bind antigen, including (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Single chain antibodies are also included by reference in the term “antibody.”

[0035] Certain therapeutic antibodies are IgG antibodies. The term “IgG” as used herein is meant a polypeptide belonging to the class of antibodies that are substantially encoded by a recognized immunoglobulin gamma gene. In humans this class comprises IgG1, IgG2, IgG3, and IgG4. In mice this class comprises IgG1, IgG2a, IgG2b, IgG3. The known Ig domains in the IgG class of antibodies are VH, C γ 1, C γ 2, C γ 3, VL, and CL. IgG is the preferred class for therapeutic antibodies for several practical reasons. IgG antibodies are stable, easily purified, and able to be stored under conditions that are practical for pharmaceutical supply chains. *In vivo* they have a long biological half-life that is not just a function of their size but is also a result of their interaction with the so-called Fc receptor (or FcRn). This receptor seems to protect IgG from catabolism within cells and recycles it back to the plasma.

[0036] Antibodies are immunological proteins that bind a specific antigen. In most mammals, including humans and mice, antibodies are constructed from paired heavy and light polypeptide chains. The light and heavy chain variable regions show significant sequence diversity between antibodies, and are responsible for binding the target antigen. Each chain is made up of individual immunoglobulin (Ig) domains, and thus the generic term immunoglobulin is used for such proteins.

[0037] The present invention includes anti-TIMP-2 antigen-binding fragments and methods of use thereof. As used herein, unless otherwise indicated, "antibody fragment" or "antigen-binding fragment" refers to antigen-binding fragments of antibodies, *i.e.* antibody fragments that retain the ability to bind specifically to the antigen bound by the full-length antibody, *e.g.* fragments that retain one or more CDR regions. Examples of antigen-binding fragments include, but are not limited to, Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules, *e.g.*, sc-Fv; nanobodies; and multispecific antibodies formed from antibody fragments (*e.g.*, bispecific antibodies, etc.).

[0038] The present invention includes anti-TIMP-2 Fab fragments and methods of use thereof. A "Fab fragment" is comprised of one light chain and the C_{H1} and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule. An "Fab fragment" can be the product of papain cleavage of an antibody.

[0039] The present invention includes anti-TIMP-2 antibodies and antigen-binding fragments thereof which comprise an Fc region and methods of use thereof. An "Fc" region contains two heavy chain fragments comprising the CH1 and CH2 domains of an antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the CH3 domains.

[0040] The present invention includes anti-TIMP-2 Fab' fragments and methods of use thereof. A "Fab' fragment" contains one light chain and a portion or fragment of one heavy chain that contains the V_H domain and the C_{H1} domain and also the region between the C_{H1} and C_{H2} domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form a F(ab')₂ molecule.

[0041] The present invention includes anti-TIMP-2 F(ab')₂ fragments and methods of use thereof. A "F(ab')₂ fragment" contains two light chains and two heavy chains containing a portion of the constant region between the C_{H1} and C_{H2} domains, such that an interchain disulfide bond is formed between the two heavy chains. A F(ab')₂ fragment thus is composed of two Fab' fragments that are held together by a disulfide bond between the two heavy chains. A "F(ab')₂ fragment" can be the product of pepsin cleavage of an antibody.

[0042] The present invention includes anti-TIMP-2 Fv fragments and methods of use thereof. The "Fv region" comprises the variable regions from both the heavy and light chains, but lacks the constant regions.

[0043] The present invention includes anti-TIMP-2 scFv fragments and methods of use thereof. The term "single-chain Fv" or "scFv" antibody refers to antibody fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen-binding. For a review of scFv, see Pluckthun (1994) *THE PHARMACOLOGY OF MONOCLONAL ANTIBODIES*, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315. See also, International Patent Application Publication No. WO 88/01649 and U.S. Pat. Nos. 4,946, 778 and 5,260,203.

[0044] The present invention includes anti-TIMP-2 domain antibodies and methods of use thereof. A "domain antibody" is an immunologically functional immunoglobulin fragment containing only the variable region of a heavy chain or the variable region of a light chain. In some instances, two or more V_H regions are covalently joined with a peptide linker to create a bivalent domain antibody. The two V_H regions of a bivalent domain antibody may target the same or different antigens.

[0045] The present invention includes anti-TIMP-2 bivalent antibodies and methods of use thereof. A "bivalent antibody" comprises two antigen-binding sites. In some instances, the two binding sites have the same antigen specificities. However, bivalent antibodies may be bispecific (see below).

[0046] The present invention includes anti-TIMP-2 camelized single domain antibodies and methods of use thereof. In certain embodiments, antibodies herein also include camelized single domain antibodies. *See, e.g.,* Muyldermans *et al.* (2001) *Trends Biochem. Sci.* 26:230; Reichmann *et al.* (1999) *J. Immunol. Methods* 231:25; WO 94/04678; WO 94/25591; U.S. Pat. No. 6,005,079). In one embodiment, the present invention provides single domain antibodies comprising two V_H domains with modifications such that single domain antibodies are formed.

[0047] The present invention includes anti-TIMP-2 diabodies and methods of use thereof. As used herein, the term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H)

connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L or V_L - V_H). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, *e.g.*, EP 404,097; WO 93/11161; and Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 6444-6448. For a review of engineered antibody variants generally see Holliger and Hudson (2005) *Nat. Biotechnol.* 23:1126-1136.

[0048] The term “specifically binds” is not intended to indicate that an antibody binds exclusively to its intended target since, as noted above, an antibody binds to any polypeptide displaying the epitope(s) to which the antibody binds. Rather, an antibody “specifically binds” if its affinity for its intended target is about 5-fold greater when compared to its affinity for a non-target molecule which does not display the appropriate epitope(s). Preferably the affinity of the antibody will be at least about 5 fold, preferably 10 fold, more preferably 25-fold, even more preferably 50-fold, and most preferably 100-fold or more, greater for a target molecule than its affinity for a non-target molecule. In preferred embodiments, Preferred antibodies bind with affinities of at least about 10^7 M^{-1} , and preferably between about 10^8 M^{-1} to about 10^9 M^{-1} , about 10^9 M^{-1} to about 10^{10} M^{-1} , or about 10^{10} M^{-1} to about 10^{12} M^{-1} .

[0049] Affinity is calculated as $K_d = k_{\text{off}}/k_{\text{on}}$ (k_{off} is the dissociation rate constant, K_{on} is the association rate constant and K_d is the equilibrium constant). Affinity can be determined at equilibrium by measuring the fraction bound (r) of labeled ligand at various concentrations (c). The data are graphed using the Scatchard equation: $r/c = K(n-r)$: where r = moles of bound ligand/mole of receptor at equilibrium; c = free ligand concentration at equilibrium; K = equilibrium association constant; and n = number of ligand binding sites per receptor molecule. By graphical analysis, r/c is plotted on the Y-axis versus r on the X-axis, thus producing a Scatchard plot. Antibody affinity measurement by Scatchard analysis is well known in the art. *See, e.g.*, van Erp *et al.*, *J. Immunoassay* 12: 425-43, 1991; Nelson and Griswold, *Comput. Methods Programs Biomed.* 27: 65-8, 1988.

[0050] Antibodies of the invention may be further characterized by epitope mapping, so that antibodies and epitopes may be selected that have the greatest clinical utility in the immunoassays described herein. The term “epitope” refers to an antigenic determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have

specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. Preferably, an epitope is targeted which is present on the target molecule, but is partially or totally absent on non-target molecules.

[0051] In some embodiments, the antibody scaffold can be a mixture of sequences from different species. As such, if the antibody is an antibody, such antibody may be a chimeric antibody and/or a humanized antibody. In general, both “chimeric antibodies” and “humanized antibodies” refer to antibodies that combine regions from more than one species. For example, “chimeric antibodies” traditionally comprise variable region(s) from a mouse (or rat, in some cases) and the constant region(s) from a human. “Humanized antibodies” generally refer to non-human antibodies that have had the variable-domain framework regions swapped for sequences found in human antibodies. Generally, in a humanized antibody, the entire antibody, except the CDRs, is encoded by a polynucleotide of human origin or is identical to such an antibody except within its CDRs. The CDRs, some or all of which are encoded by nucleic acids originating in a non-human organism, are grafted into the beta-sheet framework of a human antibody variable region to create an antibody, the specificity of which is determined by the engrafted CDRs. The creation of such antibodies is described in, e.g., WO 92/11018, Jones, 1986, Nature 321:522-525, Verhoeyen et al., 1988, Science 239:1534-1536. “Backmutation” of selected acceptor framework residues to the corresponding donor residues is often required to regain affinity that is lost in the initial grafted construct (U.S. Pat. No. 5,530,101; U.S. Pat. No. 5,585,089; U.S. Pat. No. 5,693,761; U.S. Pat. No. 5,693,762; U.S. Pat. No. 6,180,370; U.S. Pat. No. 5,859,205; U.S. Pat. No. 5,821,337; U.S. Pat. No. 6,054,297; U.S. Pat. No. 6,407,213). The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region, typically that of a human immunoglobulin, and thus will typically comprise a human Fc region. Humanized antibodies can also be generated using mice with a genetically engineered immune system. Roque et al., 2004, Biotechnol. Prog. 20:639-654. A variety of techniques and methods for humanizing and reshaping non-human antibodies are well known in the art (See Tsurushita & Vasquez, 2004, Humanization of Monoclonal Antibodies, Molecular Biology of B Cells, 533-545, Elsevier Science (USA), and references cited therein). Humanization methods include but are not limited to methods described in Jones et al.,

1986, Nature 321:522-525; Riechmann et al., 1988; Nature 332:323-329; Verhoeven et al., 1988, Science, 239:1534-1536; Queen et al., 1989, Proc Natl Acad Sci, USA 86:10029-33; He et al., 1998, J. Immunol. 160: 1029-1035; Carter et al., 1992, Proc Natl Acad Sci USA 89:4285-9, Presta et al., 1997, Cancer Res. 57(20):4593-9; Gorman et al., 1991, Proc. Natl. Acad. Sci. USA 88:4181-4185; O'Connor et al., 1998, Protein Eng 11:321-8. Humanization or other methods of reducing the immunogenicity of nonhuman antibody variable regions may include resurfacing methods, as described for example in Roguska et al., 1994, Proc. Natl. Acad. Sci. USA 91:969-973. In one embodiment, the parent antibody has been affinity matured, as is known in the art. Structure-based methods may be employed for humanization and affinity maturation, for example as described in U.S. Ser. No. 11/004,590. Selection based methods may be employed to humanize and/or affinity mature antibody variable regions, including but not limited to methods described in Wu et al., 1999, J. Mol. Biol. 294:151-162; Baca et al., 1997, J. Biol. Chem. 272(16):10678-10684; Rosok et al., 1996, J. Biol. Chem. 271(37): 22611-22618; Rader et al., 1998, Proc. Natl. Acad. Sci. USA 95: 8910-8915; Krauss et al., 2003, Protein Engineering 16(10):753-759. Other humanization methods may involve the grafting of only parts of the CDRs, including but not limited to methods described in U.S. Ser. No. 09/810,502; Tan et al., 2002, J. Immunol. 169:1119-1125; De Pascalis et al., 2002, J. Immunol. 169:3076-3084.

[0052] In one embodiment, the antibody is a fully human antibody. “Fully human antibody” or “complete human antibody” refers to a human antibody having the gene sequence of an antibody derived from a human chromosome. Fully human antibodies may be obtained, for example, using transgenic mice (Bruggemann et al., 1997, Curr Opin Biotechnol 8:455-458) or human antibody libraries coupled with selection methods (Griffiths et al., 1998, Curr Opin Biotechnol 9:102-108).

[0053] Production of Antibodies

[0054] Monoclonal antibody preparations can be produced using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS, pp. 563-681 (Elsevier, N.Y., 1981) (both

of which are incorporated by reference in their entireties). The term “monoclonal antibody” as used herein is not limited to antibodies produced through hybridoma technology. The term “monoclonal antibody” refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0055] Monoclonal antibodies derived from animals other than rats and mice offer unique advantages. Many protein targets relevant to signal transduction and disease are highly conserved between mice, rats and humans, and can therefore be recognized as self-antigens by a mouse or rat host, making them less immunogenic. This problem may be avoided when using rabbit as a host animal. See, e.g., Rossi et al., *Am. J. Clin. Pathol.*, 124, 295-302, 2005.

[0056] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with an antigen of interest or a cell expressing such an antigen. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells. Hybridomas are selected and cloned by limiting dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding the antigen. Ascites fluid, which generally contains high levels of antibodies, can be generated by inoculating mice intraperitoneally with positive hybridoma clones.

[0057] Adjuvants that can be used in the methods of antibody generation include, but are not limited to, protein adjuvants; bacterial adjuvants, e.g., whole bacteria (BCG, *Corynebacterium parvum*, *Salmonella minnesota*) and bacterial components including cell wall skeleton, trehalose dimycolate, monophosphoryl lipid A, methanol extractable residue (MER) of tubercle bacillus, complete or incomplete Freund's adjuvant; viral adjuvants; chemical adjuvants, e.g., aluminum hydroxide, iodoacetate and cholesteryl hemisuccinate; naked DNA adjuvants. Other adjuvants that can be used in the methods of the invention include, Cholera toxin, paropox proteins, MF-59 (Chiron Corporation; See also Bieg et al. (1999) “GAD65 And Insulin B Chain Peptide (9-23) Are Not Primary Autoantigens In The Type 1 Diabetes Syndrome Of The BB Rat,” *Autoimmunity*, 31(1):15-24, which is incorporated herein by reference), MPL® (Corixa Corporation; See also Lodmell et al. (2000) “DNA Vaccination Of Mice Against Rabies Virus: Effects Of

The Route Of Vaccination And The Adjuvant Monophosphoryl Lipid A (MPL),” Vaccine, 18: 1059-1066; Johnson et al. (1999) “3-O-Desacyl Monophosphoryl Lipid A Derivatives: Synthesis And Immunostimulant Activities,” Journal of Medicinal Chemistry, 42: 4640-4649; Baldrige et al. (1999) “Monophosphoryl Lipid A (MPL) Formulations For The Next Generation Of Vaccines,” Methods, 19: 103-107, all of which are incorporated herein by reference), RC-529 adjuvant (Corixa Corporation; the lead compound from Corixa’s aminoalkyl glucosaminide 4-phosphate (AGP) chemical library, see also www.corixa.com), and DETOX™ adjuvant (Corixa Corporation; DETOX™ adjuvant includes MPL® adjuvant (monophosphoryl lipid A) and mycobacterial cell wall skeleton; See also Eton et al. (1998) “Active Immunotherapy With Ultraviolet B-Irradiated Autologous Whole Melanoma Cells Plus DETOX In Patients With Metastatic Melanoma,” Clin. Cancer Res. 4(3):619-627; and Gupta et al. (1995) “Adjuvants For Human Vaccines—Current Status, Problems And Future Prospects,” Vaccine, 13(14): 1263-1276, both of which are incorporated herein by reference).

[0058] Numerous publications discuss the use of phage display technology to produce and screen libraries of polypeptides for binding to a selected analyte. See, e.g., Cwirla et al., Proc. Natl. Acad. Sci. USA 87, 6378-82, 1990; Devlin et al., Science 249, 404-6, 1990, Scott and Smith, Science 249, 386-88, 1990; and Ladner et al., U.S. Pat. No. 5,571,698. A basic concept of phage display methods is the establishment of a physical association between DNA encoding a polypeptide to be screened and the polypeptide. This physical association is provided by the phage particle, which displays a polypeptide as part of a capsid enclosing the phage genome which encodes the polypeptide. The establishment of a physical association between polypeptides and their genetic material allows simultaneous mass screening of very large numbers of phage bearing different polypeptides. Phage displaying a polypeptide with affinity to a target bind to the target and these phage are enriched by affinity screening to the target. The identity of polypeptides displayed from these phage can be determined from their respective genomes. Using these methods a polypeptide identified as having a binding affinity for a desired target can then be synthesized in bulk by conventional means. See, e.g., U.S. Patent No. 6,057,098, which is hereby incorporated in its entirety, including all tables, figures, and claims.

[0059] The antibodies that are generated by these methods may then be selected by first screening for affinity and specificity with the purified polypeptide of interest and, if

required, comparing the results to the affinity and specificity of the antibodies with polypeptides that are desired to be excluded from binding. The screening procedure can involve immobilization of the purified polypeptides in separate wells of microtiter plates. The solution containing a potential antibody or groups of antibodies is then placed into the respective microtiter wells and incubated for about 30 min to 2 h. The microtiter wells are then washed and a labeled secondary antibody (for example, an anti-mouse antibody conjugated to alkaline phosphatase if the raised antibodies are mouse antibodies) is added to the wells and incubated for about 30 min and then washed. Substrate is added to the wells and a color reaction will appear where antibody to the immobilized polypeptide(s) are present.

[0060] The antibodies so identified may then be further analyzed for affinity and specificity in the assay design selected. In the development of immunoassays for a target protein, the purified target protein acts as a standard with which to judge the sensitivity and specificity of the immunoassay using the antibodies that have been selected. Because the binding affinity of various antibodies may differ; certain antibody pairs (e.g., in sandwich assays) may interfere with one another sterically, etc., assay performance of an antibody may be a more important measure than absolute affinity and specificity of an antibody.

[0061] Antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized using conventional methodologies with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the

antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg et al. (1995) "Human Antibodies From Transgenic Mice," *Int. Rev. Immunol.* 13:65-93, which is incorporated herein by reference in its entirety). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., International Publication Nos. WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Pat. Nos. 5,413,923, 5,625,126, 5,633,425, 5,569,825, 5,661,016, 5,545,806, 5,814,318, and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.) and Medarex (Princeton, N.J.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[0062] Recombinant Expression of Antibodies

[0063] Once a nucleic acid sequence encoding an antibody of the invention has been obtained, the vector for the production of the antibody may be produced by recombinant DNA technology using techniques well known in the art. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. (See, for example, the techniques described in Sambrook et al, 1990, *MOLECULAR CLONING, A LABORATORY MANUAL*, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. and Ausubel et al. eds., 1998, *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John Wiley & Sons, NY).

[0064] An expression vector comprising the nucleotide sequence of an antibody can be transferred to a host cell by conventional techniques (e.g., electroporation, liposomal transfection, and calcium phosphate precipitation) and the transfected cells are then cultured by conventional techniques to produce the antibody of the invention. In specific embodiments, the expression of the antibody is regulated by a constitutive, an inducible or a tissue, specific promoter.

[0065] Eukaryotic and prokaryotic host cells, including mammalian cells as hosts for expression of the antibodies or fragments or immunoglobulin chains disclosed herein are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, *inter alia*, Chinese hamster ovary (CHO) cells, NS0, SP2 cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.*, Hep G2), A549 cells, 3T3 cells, HEK-293 cells and a number of other cell lines. Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse and hamster cells. Cell lines of particular preference are selected through determining which cell lines have high expression levels. Other cell lines that may be used are insect cell lines, such as Sf9 cells, amphibian cells, bacterial cells, plant cells and fungal cells. Fungal cells include yeast and filamentous fungus cells including, for example, *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia sp.*, *Saccharomyces cerevisiae*, *Saccharomyces sp.*, *Hansenula polymorpha*, *Kluyveromyces sp.*, *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Trichoderma reesei*, *Chrysosporium lucknowense*, *Fusarium sp.*, *Fusarium gramineum*, *Fusarium venenatum*, *Physcomitrella patens* and *Neurospora crassa*. *Pichia sp.*, any *Saccharomyces sp.*, *Hansenula polymorpha*, any *Kluyveromyces sp.*, *Candida albicans*, any *Aspergillus sp.*, *Trichoderma reesei*, *Chrysosporium lucknowense*, any *Fusarium sp.*, *Yarrowia lipolytica*, and *Neurospora crassa*. When recombinant expression vectors encoding the heavy chain or antigen-binding portion or fragment thereof, the light chain and/or antigen-binding fragment thereof are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody or fragment or chain in the host cells or secretion of the into the culture medium in which the host cells are grown.

[0066] A variety of host-expression vector systems may be utilized to express the antibodies of the invention. Such host-expression systems represent vehicles by which the coding sequences of the antibodies may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the antibodies of the invention *in situ*. These

include, but are not limited to, microorganisms such as bacteria (e.g., *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing immunoglobulin coding sequences; yeast (e.g., *Saccharomyces pichia*) transformed with recombinant yeast expression vectors containing immunoglobulin coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the immunoglobulin coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing immunoglobulin coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 293T, 3T3 cells, lymphotic cells (see U.S. Pat. No. 5,807,715), Per C.6 cells (rat retinal cells developed by Crucell)) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

[0067] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al. (1983) "Easy Identification Of cDNA Clones," EMBO J. 2:1791-1794), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye et al. (1985) "Up-Promoter Mutations In The Lpp Gene Of Escherichia coli," Nucleic Acids Res. 13:3101-3110; Van Heeke et al. (1989) "Expression Of Human Asparagine Synthetase In Escherichia coli," J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[0068] In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (e.g., the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (e.g., the polyhedrin promoter).

[0069] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts. (see e.g., see Logan et al. (1984) "Adenovirus Tripartite Leader Sequence Enhances Translation Of mRNAs Late After Infection," *Proc. Natl. Acad. Sci. (U.S.A.)* 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bitter et al. (1987) "Expression And Secretion Vectors For Yeast," *Methods in Enzymol.* 153:516-544).

[0070] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO,

VERY, BHK, Hela, COS, MDCK, 293, 293T, 3T3, WI38, BT483, Hs578T, HTB2, BT20 and T47D, CRL7030 and Hs578Bst.

[0071] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express an antibody of the invention may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibodies of the invention. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibodies of the invention.

[0072] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al. (1977) "Transfer Of Purified Herpes Virus Thymidine Kinase Gene To Cultured Mouse Cells," Cell 11:223-232), hypoxanthine-guanine phosphoribosyltransferase (Szybalska et al. (1962) "Genetics Of Human Cess Line. IV. DNA-Mediated Heritable Transformation Of A Biochemical Trait," Proc. Natl. Acad. Sci. (U.S.A.) 48:2026-2034), and adenine phosphoribosyltransferase (Lowy et al. (1980) "Isolation Of Transforming DNA: Cloning The Hamster Aprt Gene," Cell 22:817-823) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al. (1980) "Transformation Of Mammalian Cells With An Amplifiable Dominant-Acting Gene," Proc. Natl. Acad. Sci. (U.S.A.) 77:3567-3570; O'Hare et al. (1981) "Transformation Of Mouse Fibroblasts To Methotrexate Resistance By A Recombinant Plasmid Expressing A Prokaryotic Dihydrofolate Reductase," Proc. Natl. Acad. Sci. (U.S.A.) 78:1527-1531); gpt, which confers resistance to mycophenolic acid (Mulligan et al. (1981) "Selection For Animal Cells That Express The Escherichia coli Gene Coding For Xanthine-Guanine Phosphoribosyltransferase," Proc. Natl. Acad. Sci. (U.S.A.) 78:2072-2076); neo, which

confers resistance to the aminoglycoside G-418 (Tachibana et al. (1991) "Altered Reactivity Of Immunoglobulin Produced By Human-Human Hybridoma Cells Transfected By pSV2-Neo Gene," *Cytotechnology* 6(3):219-226; Tolstoshev (1993) "Gene Therapy, Concepts, Current Trials And Future Directions," *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan (1993) "The Basic Science Of Gene Therapy," *Science* 260:926-932; and Morgan et al. (1993) "Human gene therapy," *Ann. Rev. Biochem.* 62:191-217). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John Wiley & Sons, NY; Kriegler, 1990, *GENE TRANSFER AND EXPRESSION, A LABORATORY MANUAL*, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, *CURRENT PROTOCOLS IN HUMAN GENETICS*, John Wiley & Sons, NY.; Colbere-Garapin et al. (1981) "A New Dominant Hybrid Selective Marker For Higher Eukaryotic Cells," *J. Mol. Biol.* 150:1-14; and hygromycin, which confers resistance to hygromycin (Santerre et al. (1984) "Expression Of Prokaryotic Genes For Hygromycin B And G418 Resistance As Dominant-Selection Markers In Mouse L Cells," *Gene* 30:147-156).

[0073] The expression levels of an antibody of the invention can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use Of Vectors Based On Gene Amplification For The Expression Of Cloned Genes In Mammalian Cells," in *DNA CLONING*, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing an antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of the antibody, production of the antibody will also increase (Crouse et al. (1983) "Expression And Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes," *Mol. Cell. Biol.* 3:257-266).

[0074] The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot (1986) "Expression And

Amplification Of Engineered Mouse Dihydrofolate Reductase Minigenes,” Nature 322:562-565; Kohler (1980) “Immunoglobulin Chain Loss In Hybridoma Lines,” Proc. Natl. Acad. Sci. (U.S.A.) 77:2197-2199). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

[0075] In general, glycoproteins produced in a particular cell line or transgenic animal will have a glycosylation pattern that is characteristic for glycoproteins produced in the cell line or transgenic animal. Therefore, the particular glycosylation pattern of an antibody will depend on the particular cell line or transgenic animal used to produce the antibody. However, all antibodies encoded by the nucleic acid molecules provided herein, or comprising the amino acid sequences provided herein, comprise the instant invention, independent of the glycosylation pattern the antibodies may have. Similarly, in particular embodiments, antibodies with a glycosylation pattern comprising only non-fucosylated *N*-glycans may be advantageous, because these antibodies have been shown to typically exhibit more potent efficacy than their fucosylated counterparts both *in vitro* and *in vivo* (See for example, Shinkawa *et al.*, *J. Biol. Chem.* 278: 3466-3473 (2003); U.S. Patent Nos. 6,946,292 and 7,214,775). These antibodies with non-fucosylated *N*-glycans are not likely to be immunogenic because their carbohydrate structures are a normal component of the population that exists in human serum IgG.

[0076] Once the antibody of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an antibody, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

[0077] Diagnosis of Acute Renal Failure

[0078] The present invention relates in part to administration of anti-TIMP-2 antibodies to patients diagnosed with AKI. As noted above, the terms “acute renal (or kidney) injury” and “acute renal (or kidney) failure” as used herein are defined in part in terms of changes in serum creatinine from a baseline value. Most definitions of ARF have common elements, including the use of serum creatinine and, often, urine output. Patients may present with renal dysfunction without an available baseline measure of renal function for use in this comparison. In such an event, one may estimate a baseline serum creatinine value by assuming the patient initially had a normal GFR. Glomerular filtration

rate (GFR) is the volume of fluid filtered from the renal (kidney) glomerular capillaries into the Bowman's capsule per unit time. Glomerular filtration rate (GFR) can be calculated by measuring any chemical that has a steady level in the blood, and is freely filtered but neither reabsorbed nor secreted by the kidneys. GFR is typically expressed in units of ml/min:

$$GFR = \frac{\text{Urine Concentration} \times \text{Urine Flow}}{\text{Plasma Concentration}}$$

[0079] By normalizing the GFR to the body surface area, a GFR of approximately 75–100 ml/min per 1.73 m² can be assumed. The rate therefore measured is the quantity of the substance in the urine that originated from a calculable volume of blood.

[0080] There are several different techniques used to calculate or estimate the glomerular filtration rate (GFR or eGFR). In clinical practice, however, creatinine clearance is used to measure GFR. Creatinine is produced naturally by the body (creatinine is a metabolite of creatine, which is found in muscle). It is freely filtered by the glomerulus, but also actively secreted by the renal tubules in very small amounts such that creatinine clearance overestimates actual GFR by 10-20%. This margin of error is acceptable considering the ease with which creatinine clearance is measured.

[0081] Creatinine clearance (CCr) can be calculated if values for creatinine's urine concentration (U_{Cr}), urine flow rate (V), and creatinine's plasma concentration (P_{Cr}) are known. Since the product of urine concentration and urine flow rate yields creatinine's excretion rate, creatinine clearance is also said to be its excretion rate (U_{Cr}×V) divided by its plasma concentration. This is commonly represented mathematically as:

$$CCr = \frac{U_{Cr} \times V}{P_{Cr}}$$

[0082] Commonly a 24 hour urine collection is undertaken, from empty-bladder one morning to the contents of the bladder the following morning, with a comparative blood test then taken:

$$CCr = \frac{U_{Cr} \times 24\text{-hour volume}}{P_{Cr} \times 24 \times 60\text{mins}}$$

[0083] To allow comparison of results between people of different sizes, the CCr is often corrected for the body surface area (BSA) and expressed compared to the average

sized man as ml/min/1.73 m². While most adults have a BSA that approaches 1.7 (1.6-1.9), extremely obese or slim patients should have their CCr corrected for their actual BSA:

$$C_{Cr \text{ corrected}} = \frac{C_{Cr} \times 1.73}{BSA}$$

[0084] The accuracy of a creatinine clearance measurement (even when collection is complete) is limited because as glomerular filtration rate (GFR) falls creatinine secretion is increased, and thus the rise in serum creatinine is less. Thus, creatinine excretion is much greater than the filtered load, resulting in a potentially large overestimation of the GFR (as much as a twofold difference). However, for clinical purposes it is important to determine whether renal function is stable or getting worse or better. This is often determined by monitoring serum creatinine alone. Like creatinine clearance, the serum creatinine will not be an accurate reflection of GFR in the non-steady-state condition of ARF. Nonetheless, the degree to which serum creatinine changes from baseline will reflect the change in GFR. Serum creatinine is readily and easily measured and it is specific for renal function.

[0085] For purposes of determining urine output on a Urine output on a mL/kg/hr basis, hourly urine collection and measurement is adequate. In the case where, for example, only a cumulative 24-h output was available and no patient weights are provided, minor modifications of the RIFLE urine output criteria have been described. For example, Bagshaw et al., *Nephrol. Dial. Transplant.* 23: 1203–1210, 2008, assumes an average patient weight of 70 kg, and patients are assigned a RIFLE classification based on the following: <35 mL/h (Risk), <21 mL/h (Injury) or <4 mL/h (Failure).

[0086] Risk Assessment

[0087] Because AKI is associated with significant morbidity and mortality, and because no specific treatment is available to reverse AKI, early recognition and management is paramount. Indeed, recognition of patients at risk for AKI, or with possible AKI but prior to clinical manifestations, is likely to result in better outcomes than treating only established AKI. Thus, the present invention relates in part to administration of anti-TIMP-2 antibodies to patients at high risk of imminent (within 72 hours, and more preferably 48, 24, 18, or 12 hours) AKI.

[0100] High-risk patients include those with risk factors for acute kidney injury (AKI) but have normal GFR. For example, they may include patients who have diabetes and hypertension or are taking medications such as nonsteroidal anti-inflammatory drugs or angiotensin-converting enzyme inhibitors. These individuals represent a population that needs to be identified to modify risk factors if possible and initiate preventive strategies when indicated (e.g., contrast studies). Patients with prerenal AKI are those with prerenal urinary indices and failure of autoregulatory mechanisms that lead to a decrease in GFR (e.g., dehydration). These individuals have the potential for rapid reversal of their prerenal condition. During this period, injury to brush border of proximal tubule cells may be present but undetectable. However, with novel biomarkers, identification of early injury and treatment is possible. Patients with AKI may represent an extension from severe prerenal AKI. Alternatively, in some conditions, AKI may not be preceded by a prerenal state (e.g., sepsis, exposure to nephrotoxins). Serum creatinine is a late marker and will detect AKI after substantial injury is present. At this point, intervention may be too late.

[0101] The KDIGO Clinical Practice Guideline for Acute Kidney Injury (Kidney Intl. 2 (Suppl 1), 2012, which is incorporated by reference in its entirety including all appendices, provides a description of risk assessment for AKI, particularly in Chapter 2.2 and Appendix D. Risk factors include hydration state, hypoalbuminemia, advanced age, female gender, black race, presence of CKD, diabetes, heart disease, sepsis, or pulmonary disease, exposure to certain medications and contrast agents that are nephrotoxic, cardiac surgery, etc.

[0102] Use of Biomarkers

[0103] Serum creatinine measurement (SCr) is the gold-standard marker for renal function. However, SCr concentrations may be inaccurate in detecting an abrupt decline in renal function, as the functional reserve of the remaining healthy nephrons prevents a significant rise in SCr until 50% of nephrons are lost. Thus, because SCr is a “late” marker of renal injury, even if the SCr-based estimation of renal function is “normal” renal injury may already have begun. Other biomarkers which have been used to assess AKI include serum and urinary Cystatin C, serum and urinary neutrophil gelatinase-associated lipocalin, urinary Kidney Injury Molecule 1, Interleukin-18, Liver-type fatty acid binding protein, and N-acetyl- β -D-glucosaminidase.

[0104] In 2014, the US Food and Drug Administration approved the marketing of a test based on the combination of urine concentrations of tissue inhibitor of metalloproteinase 2 and insulin-like growth factor binding protein 7 ([TIMP-2] × [IGFBP7]) to identify patients are at risk for developing future AKI. In one study, a single measurement of urinary TIMP2•IGFBP7 soon after ICU admission and found an AUC-ROC of 0.84 for development of moderate-to-severe AKI within 12 hours. Using a cutoff value of 0.3, the test showed a sensitivity and specificity of 89% (77–97%) and 49% (43–54%), respectively; at a cutoff value of 2.0 the test showed a sensitivity and specificity of 40% (23–57%) and 94% (92–96%). Gunnerson et al., J. Trauma Acute Care Surg. 80: 243–49, 2016. Such tests provide an ideal method of identifying high risk patients for treatment according to the methods of the present invention.

[0105] Pharmaceutical Compositions and Administration

[0088] To prepare pharmaceutical or sterile compositions of the anti-TIMP-2 antibodies and antigen-binding fragments of the invention, the antibody or antigen-binding fragment thereof is admixed with a pharmaceutically acceptable carrier or excipient. See, *e.g.*, *Remington's Pharmaceutical Sciences* and *U.S. Pharmacopeia: National Formulary*, Mack Publishing Company, Easton, PA (1984).

[0089] Formulations of therapeutic and diagnostic agents may be prepared by mixing with acceptable carriers, excipients, or stabilizers in the form of, *e.g.*, lyophilized powders, slurries, aqueous solutions or suspensions (see, *e.g.*, Hardman, *et al.* (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, NY; Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, Lippincott, Williams, and Wilkins, New York, NY; Avis, *et al.* (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Marcel Dekker, NY; Weiner and Kotkoskie (2000) *Excipient Toxicity and Safety*, Marcel Dekker, Inc., New York, NY).

[0090] Toxicity and therapeutic efficacy of the antibodies of the invention, administered alone or in combination with another therapeutic agent, can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose

therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index (LD_{50}/ED_{50}). The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration.

[0091] In a further embodiment, a further therapeutic agent that is administered to a subject in association with an anti-TIMP-2 antibody or antigen-binding fragment thereof of the invention in accordance with the Physicians' Desk Reference 2003 (Thomson Healthcare; 57th edition (November 1, 2002)).

[0092] The mode of administration can vary. Routes of administration include oral, rectal, transmucosal, intestinal, parenteral; intramuscular, subcutaneous, intradermal, intramedullary, intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, intraocular, inhalation, insufflation, topical, cutaneous, transdermal, or intra-arterial.

[0093] In particular embodiments, the anti-TIMP-2 antibodies or antigen-binding fragments thereof of the invention can be administered by an invasive route such as by injection. In further embodiments of the invention, an anti-TIMP-2 antibody or antigen-binding fragment thereof, or pharmaceutical composition thereof, is administered intravenously, subcutaneously, intramuscularly, intraarterially, or by inhalation, aerosol delivery. Administration by non-invasive routes (*e.g.*, orally; for example, in a pill, capsule or tablet) is also within the scope of the present invention.

[0094] The present invention provides a vessel (*e.g.*, a plastic or glass vial, *e.g.*, with a cap or a chromatography column, hollow bore needle or a syringe cylinder) comprising any of the antibodies or antigen-binding fragments of the invention or a pharmaceutical composition thereof. The present invention also provides an injection device comprising any of the antibodies or antigen-binding fragments of the invention or a pharmaceutical composition thereof. An injection device is a device that introduces a substance into the body of a patient via a parenteral route, *e.g.*, intramuscular, subcutaneous or intravenous. For example, an injection device may be a syringe (*e.g.*, pre-filled with the pharmaceutical composition, such as an auto-injector) which, for example, includes a cylinder or barrel for holding fluid to be injected (*e.g.*, antibody or fragment or a

pharmaceutical composition thereof), a needle for piecing skin and/or blood vessels for injection of the fluid; and a plunger for pushing the fluid out of the cylinder and through the needle bore. In an embodiment of the invention, an injection device that comprises an antibody or antigen-binding fragment thereof of the present invention or a pharmaceutical composition thereof is an intravenous (IV) injection device. Such a device includes the antibody or fragment or a pharmaceutical composition thereof in a cannula or trocar/needle which may be attached to a tube which may be attached to a bag or reservoir for holding fluid (*e.g.*, saline; or lactated ringer solution comprising NaCl, sodium lactate, KCl, CaCl₂ and optionally including glucose) introduced into the body of the patient through the cannula or trocar/needle. The antibody or fragment or a pharmaceutical composition thereof may, in an embodiment of the invention, be introduced into the device once the trocar and cannula are inserted into the vein of a subject and the trocar is removed from the inserted cannula. The IV device may, for example, be inserted into a peripheral vein (*e.g.*, in the hand or arm); the superior vena cava or inferior vena cava, or within the right atrium of the heart (*e.g.*, a central IV); or into a subclavian, internal jugular, or a femoral vein and, for example, advanced toward the heart until it reaches the superior vena cava or right atrium (*e.g.*, a central venous line). In an embodiment of the invention, an injection device is an autoinjector; a jet injector or an external infusion pump. A jet injector uses a high-pressure narrow jet of liquid which penetrate the epidermis to introduce the antibody or fragment or a pharmaceutical composition thereof to a patient's body. External infusion pumps are medical devices that deliver the antibody or fragment or a pharmaceutical composition thereof into a patient's body in controlled amounts. External infusion pumps may be powered electrically or mechanically. Different pumps operate in different ways, for example, a syringe pump holds fluid in the reservoir of a syringe, and a moveable piston controls fluid delivery, an elastomeric pump holds fluid in a stretchable balloon reservoir, and pressure from the elastic walls of the balloon drives fluid delivery. In a peristaltic pump, a set of rollers pinches down on a length of flexible tubing, pushing fluid forward. In a multi-channel pump, fluids can be delivered from multiple reservoirs at multiple rates.

[0095] The pharmaceutical compositions disclosed herein may also be administered with a needleless hypodermic injection device; such as the devices disclosed in U.S. Patent Nos. 6,620,135; 6,096,002; 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556. Such needleless devices comprising the

pharmaceutical composition are also part of the present invention. The pharmaceutical compositions disclosed herein may also be administered by infusion. Examples of well-known implants and modules for administering the pharmaceutical compositions include those disclosed in: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments. Many other such implants, delivery systems, and modules are well known to those skilled in the art and those comprising the pharmaceutical compositions of the present invention are within the scope of the present invention.

[0096] The administration regimen depends on several factors, including the serum or tissue turnover rate of the therapeutic antibody or antigen-binding fragment, the level of symptoms, the immunogenicity of the therapeutic antibody, and the accessibility of the target cells in the biological matrix. Preferably, the administration regimen delivers sufficient therapeutic antibody or fragment to effect improvement in the target disease state, while simultaneously minimizing undesired side effects. Accordingly, the amount of biologic delivered depends in part on the particular therapeutic antibody and the severity of the condition being treated. Guidance in selecting appropriate doses of therapeutic antibodies or fragments is available (see, *e.g.*, Wawrzynczak (1996) *Antibody Therapy*, Bios Scientific Pub. Ltd, Oxfordshire, UK; Kresina (ed.) (1991) *Monoclonal Antibodies, Cytokines and Arthritis*, Marcel Dekker, New York, NY; Bach (ed.) (1993) *Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases*, Marcel Dekker, New York, NY; Baert, *et al.* (2003) *New Engl. J. Med.* 348:601-608; Milgrom *et al.* (1999) *New Engl. J. Med.* 341:1966-1973; Slamon *et al.* (2001) *New Engl. J. Med.* 344:783-792; Beniaminovitz *et al.* (2000) *New Engl. J. Med.* 342:613-619; Ghosh *et al.* (2003) *New Engl. J. Med.* 348:24-32; Lipsky *et al.* (2000) *New Engl. J. Med.* 343:1594-1602).

[0097] Determination of the appropriate dose is made by the clinician, *e.g.*, using parameters or factors known or suspected in the art to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any

negative side effects. Important diagnostic measures include those of symptoms of, *e.g.*, the inflammation or level of inflammatory cytokines produced. In general, it is desirable that a biologic that will be used is derived from the same species as the animal targeted for treatment, thereby minimizing any immune response to the reagent. In the case of human subjects, for example, humanized and fully human antibodies are may be desirable.

[0098] Antibodies or antigen-binding fragments thereof disclosed herein may be provided by continuous infusion, or by doses administered, *e.g.*, daily, 1-7 times per week, weekly, bi-weekly, monthly, bimonthly, quarterly, semiannually, annually etc. Doses may be provided, *e.g.*, intravenously, subcutaneously, topically, orally, nasally, rectally, intramuscular, intracerebrally, intraspinally, or by inhalation. A total weekly dose is generally at least 0.05 µg/kg body weight, more generally at least 0.2 µg/kg, 0.5 µg/kg, 1 µg/kg, 10 µg/kg, 100 µg/kg, 0.25 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 5.0 mg/ml, 10 mg/kg, 25 mg/kg, 50 mg/kg or more (see, *e.g.*, Yang, *et al.* (2003) *New Engl. J. Med.* 349:427-434; Herold, *et al.* (2002) *New Engl. J. Med.* 346:1692-1698; Liu, *et al.* (1999) *J. Neurol. Neurosurg. Psych.* 67:451-456; Portielji, *et al.* (20003) *Cancer Immunol. Immunother.* 52:151-144). Doses may also be provided to achieve a pre-determined target concentration of anti-TIMP-2 antibody in the subject's serum, such as 0.1, 0.3, 1, 3, 10, 30, 100, 300 µg/ml or more. In other embodiments, An anti-TIMP-2 antibody of the present invention is administered, *e.g.*, subcutaneously or intravenously, on a weekly, biweekly, "every 4 weeks," monthly, bimonthly, or quarterly basis at 10, 20, 50, 80, 100, 200, 500, 1000 or 2500 mg/subject.

[0099] As used herein, the term "effective amount" refer to an amount of an anti-TIMP-2 or antigen-binding fragment thereof of the invention that, when administered alone or in combination with an additional therapeutic agent to a cell, tissue, or subject, is effective to cause a measurable improvement in one or more symptoms of disease, for example cancer or the progression of cancer. An effective dose further refers to that amount of the antibody or fragment sufficient to result in at least partial amelioration of symptoms, *e.g.*, improved renal function or histology. When applied to an individual active ingredient administered alone, an effective dose refers to that ingredient alone. When applied to a combination, an effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. An effective amount of a therapeutic will result

in an improvement of a diagnostic measure or parameter by at least 10%; usually by at least 20%; preferably at least about 30%; more preferably at least 40%, and most preferably by at least 50%. An effective amount can also result in an improvement in a subjective measure in cases where subjective measures are used to assess disease severity.

[00100] Additional Treatment Modalities

[00101] Once a diagnosis is obtained or a patient is identified as “high risk”, the clinician can readily select a treatment regimen that is compatible with the diagnosis, such as initiating renal replacement therapy, withdrawing delivery of compounds that are known to be damaging to the kidney, kidney transplantation, delaying or avoiding procedures that are known to be damaging to the kidney, modifying diuretic administration, initiating goal directed therapy, etc. The skilled artisan is aware of appropriate treatments for numerous diseases discussed in relation to the methods of diagnosis described herein. See, e.g., Merck Manual of Diagnosis and Therapy, 17th Ed. Merck Research Laboratories, Whitehouse Station, NJ, 1999. These treatments may be used together with the administration of anti-TIMP-2 antibodies according to the present invention.

[00102] Management and treatment options for AKI are multifactorial and ultimately must be tailored to each specific situation and patient. As previously stated, RRT, whether continuous or intermittent, is a blood purification system that substitutes for kidney function. However, RRT is associated with risks and complications, as discussed above, and evidence suggests that RRT may be an independent risk factor for worse outcomes. From a risk-benefit perspective, RRT should be reserved for those patients deemed most likely to have persistent AKI and/or most likely not to recover. In this dichotomous construct, conservative management frequently represents the preferred clinical choice for many common clinical scenarios, including situations where clinical evidence, bedside evaluation, and objective data suggest a high likelihood for recovery from and/or non-persistence of AKI.

[00103] Conservative management is defined as medical interventions and approaches that address the dangerous and life-threatening manifestations of AKI without the use of RRT. These management strategies include – but are not limited to – the key physiological and pathophysiological disturbances such as hypervolemia and fluid imbalances, acidosis and acid-base disorders, electrolyte disturbances (e.g.

hyperkalemia), and uremia and severe azotemia. Conservative management strategies consist of the following basic strategies:

Hemodynamic support:

- a. Fluid therapy, including the use of crystalloids, and colloids
- b. Administration of vasopressor and vasoactive agents (commonly used vasopressor agents include the following: Norepinephrine, Epinephrine, Phenylephrine, Dopamine, Vasopressin)
- c. Diuretic therapy, including the use of loop and osmotic diuretics
- d. Treatment of acidosis and other acid-base abnormalities
- e. Treatment of potassium and other electrolyte disturbances and imbalances.
- f. Glycemic control and treatment of hyperglycemia.
- g. Nutritional support.

[00104] Hyperkalemia, when severe, is a medical emergency and requires immediate intervention. Treatment approaches are divided into three main approaches: 1) Interventions that cause potassium to shift from the extracellular space into the intracellular space, 2) Interventions that stabilize membrane actions of potassium, and 3) Interventions that enhance potassium elimination. These interventions are often provided concurrently.

[00105] Patients with AKI commonly develop acid-base abnormalities, most notably metabolic acidosis, which requires intervention when clinically significant.

[00106] Elevated glucose due to stress hyperglycemia is frequently encountered in AKI and other critical illnesses, and while evidence remains controversial with respect to outcome benefit associated glycemic control, patients and populations likely to benefit, and desired glucose targets and the specific therapeutic approaches to achieve (relative) euglycemia, consensus and current standard of care suggest the following: use of insulin therapy to achieve a serum glucose level between 110 to 149 mg/dL (6.1 - 8.3 mmol/L) and above; and frequent monitoring of serum glucose.

[00107] Beyond conservative management, physicians may determine that initiation or maintenance of renal replacement therapy is appropriate. Intermittent RRT includes

hemodialysis and sustained low-efficiency dialysis, while continuous RRT refers to ultrafiltration (UF), Continuous Venovenous Hemofiltration (CVVH), Continuous Venovenous Hemodialysis (CVVHD), Continuous Venovenous Hemodiafiltration (CVVHDF), Continuous Venovenous High-Flux Hemodialysis, and Continuous Arterial Venous Hemofiltration (CAVH).

[00108] General Methods

[00109] Standard methods in molecular biology are described Sambrook, Fritsch and Maniatis (1982 & 1989 2nd Edition, 2001 3rd Edition) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sambrook and Russell (2001) *Molecular Cloning, 3rd ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, CA). Standard methods also appear in Ausbel, *et al.* (2001) *Current Protocols in Molecular Biology, Vols.1-4*, John Wiley and Sons, Inc. New York, NY, which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4).

[00110] Methods for protein purification including immunoprecipitation, chromatography, electrophoresis, centrifugation, and crystallization are described (Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 1*, John Wiley and Sons, Inc., New York). Chemical analysis, chemical modification, post-translational modification, production of fusion proteins, glycosylation of proteins are described (see, *e.g.*, Coligan, *et al.* (2000) *Current Protocols in Protein Science, Vol. 2*, John Wiley and Sons, Inc., New York; Ausubel, *et al.* (2001) *Current Protocols in Molecular Biology, Vol. 3*, John Wiley and Sons, Inc., NY, NY, pp. 16.0.5-16.22.17; Sigma-Aldrich, Co. (2001) *Products for Life Science Research*, St. Louis, MO; pp. 45-89; Amersham Pharmacia Biotech (2001) *BioDirectory*, Piscataway, N.J., pp. 384-391). Production, purification, and fragmentation of polyclonal and monoclonal antibodies are described (Coligan, *et al.* (2001) *Current Protocols in Immunology, Vol. 1*, John Wiley and Sons, Inc., New York; Harlow and Lane (1999) *Using Antibodies*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Harlow and Lane, *supra*). Standard techniques for characterizing ligand/receptor interactions are available (see, *e.g.*, Coligan, *et al.* (2001) *Current Protocols in Immunology, Vol. 4*, John Wiley, Inc., New York).

[00111] Monoclonal, polyclonal, and humanized antibodies can be prepared (see, *e.g.*, Sheperd and Dean (eds.) (2000) *Monoclonal Antibodies*, Oxford Univ. Press, New York, NY; Kontermann and Dubel (eds.) (2001) *Antibody Engineering*, Springer-Verlag, New York; Harlow and Lane (1988) *Antibodies A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 139-243; Carpenter, *et al.* (2000) *J. Immunol.* 165:6205; He, *et al.* (1998) *J. Immunol.* 160:1029; Tang *et al.* (1999) *J. Biol. Chem.* 274:27371-27378; Baca *et al.* (1997) *J. Biol. Chem.* 272:10678-10684; Chothia *et al.* (1989) *Nature* 342:877-883; Foote and Winter (1992) *J. Mol. Biol.* 224:487-499; U.S. Pat. No. 6,329,511).

[00112] An alternative to humanization is to use human antibody libraries displayed on phage or human antibody libraries in transgenic mice (Vaughan *et al.* (1996) *Nature Biotechnol.* 14:309-314; Barbas (1995) *Nature Medicine* 1:837-839; Mendez *et al.* (1997) *Nature Genetics* 15:146-156; Hoogenboom and Chames (2000) *Immunol. Today* 21:371-377; Barbas *et al.* (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Kay *et al.* (1996) *Phage Display of Peptides and Proteins: A Laboratory Manual*, Academic Press, San Diego, CA; de Bruin *et al.* (1999) *Nature Biotechnol.* 17:397-399).

[00113] Single chain antibodies and diabodies are described (see, *e.g.*, Malecki *et al.* (2002) *Proc. Natl. Acad. Sci. USA* 99:213-218; Conrath *et al.* (2001) *J. Biol. Chem.* 276:7346-7350; Desmyter *et al.* (2001) *J. Biol. Chem.* 276:26285-26290; Hudson and Kortt (1999) *J. Immunol. Methods* 231:177-189; and U.S. Pat. No. 4,946,778). Bifunctional antibodies are provided (see, *e.g.*, Mack, *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:7021-7025; Carter (2001) *J. Immunol. Methods* 248:7-15; Volkel, *et al.* (2001) *Protein Engineering* 14:815-823; Segal, *et al.* (2001) *J. Immunol. Methods* 248:1-6; Brennan, *et al.* (1985) *Science* 229:81-83; Raso, *et al.* (1997) *J. Biol. Chem.* 272:27623; Morrison (1985) *Science* 229:1202-1207; Traunecker, *et al.* (1991) *EMBO J.* 10:3655-3659; and U.S. Pat. Nos. 5,932,448, 5,532,210, and 6,129,914).

[00114] Multispecific antibodies are also provided (see, *e.g.*, Azzoni *et al.* (1998) *J. Immunol.* 161:3493; Kita *et al.* (1999) *J. Immunol.* 162:6901; Merchant *et al.* (2000) *J. Biol. Chem.* 275:9115; Pandey *et al.* (2000) *J. Biol. Chem.* 275:38633; Zheng *et al.* (2001) *J. Biol. Chem.* 276:12999; Propst *et al.* (2000) *J. Immunol.* 165:2214; Long (1999) *Ann. Rev. Immunol.* 17:875; Labrijn *et al.*, *Proc. Natl. Acad. Sci. USA* 110: 5145-50, 2013; de Jong *et al.*, *PLOS Biol* 14(1): e1002344, 2016 (doi:10.1371/journal.pbio.1002344).

[00115] Purification of antigen is not necessary for the generation of antibodies. Animals can be immunized with cells bearing the antigen of interest. Splenocytes can then be isolated from the immunized animals, and the splenocytes can fused with a myeloma cell line to produce a hybridoma (see, *e.g.*, Meyaard *et al.* (1997) *Immunity* 7:283-290; Wright *et al.* (2000) *Immunity* 13:233-242; Preston *et al.*, *supra*; Kaithamana *et al.* (1999) *J. Immunol.* 163:5157-5164).

[00116] Antibodies can be conjugated, *e.g.*, to small drug molecules, enzymes, liposomes, polyethylene glycol (PEG). Antibodies are useful for therapeutic, diagnostic, kit or other purposes, and include antibodies coupled, *e.g.*, to dyes, radioisotopes, enzymes, or metals, *e.g.*, colloidal gold (see, *e.g.*, Le Doussal *et al.* (1991) *J. Immunol.* 146:169-175; Gibellini *et al.* (1998) *J. Immunol.* 160:3891-3898; Hsing and Bishop (1999) *J. Immunol.* 162:2804-2811; Everts *et al.* (2002) *J. Immunol.* 168:883-889).

[00117] Methods for flow cytometry, including fluorescence activated cell sorting (FACS), are available (see, *e.g.*, Owens, *et al.* (1994) *Flow Cytometry Principles for Clinical Laboratory Practice*, John Wiley and Sons, Hoboken, NJ; Givan (2001) *Flow Cytometry*, 2nd ed.; Wiley-Liss, Hoboken, NJ; Shapiro (2003) *Practical Flow Cytometry*, John Wiley and Sons, Hoboken, NJ). Fluorescent reagents suitable for modifying nucleic acids, including nucleic acid primers and probes, polypeptides, and antibodies, for use, *e.g.*, as diagnostic reagents, are available (Molecular Probes (2003) *Catalogue*, Molecular Probes, Inc., Eugene, OR; Sigma-Aldrich (2003) *Catalogue*, St. Louis, MO).

[00118] Standard methods of histology of the immune system are described (see, *e.g.*, Muller-Harmelink (ed.) (1986) *Human Thymus: Histopathology and Pathology*, Springer Verlag, New York, NY; Hiatt, *et al.* (2000) *Color Atlas of Histology*, Lippincott, Williams, and Wilkins, Phila, PA; Louis, *et al.* (2002) *Basic Histology: Text and Atlas*, McGraw-Hill, New York, NY).

[00119] Software packages and databases for determining, *e.g.*, antigenic fragments, leader sequences, protein folding, functional domains, glycosylation sites, and sequence alignments, are available (see, *e.g.*, GenBank, Vector NTI® Suite (Informax, Inc, Bethesda, MD); GCG Wisconsin Package (Accelrys, Inc., San Diego, CA); DeCypher® (TimeLogic Corp., Crystal Bay, Nevada); Menne, *et al.* (2000) *Bioinformatics* 16: 741-742; Menne, *et al.* (2000) *Bioinformatics Applications Note* 16:741-742; Wren, *et al.*

(2002) *Comput. Methods Programs Biomed.* 68:177-181; von Heijne (1983) *Eur. J. Biochem.* 133:17-21; von Heijne (1986) *Nucleic Acids Res.* 14:4683-4690).

[00120]

EXAMPLES

[00121] Example 1: Monoclonal Antibody development in rabbits

[00122] Female New Zealand Rabbits are immunized by subcutaneous injections (SQ) with antigen/adjuvant emulsions. Primary immunization is done with Complete Freund's Adjuvant and Incomplete Freund's Adjuvant is used for all subsequent boosts. Rabbits are injected SQ every three weeks at 250µg protein antigen per rabbit (alternating two sites, hips and scapulas). A test bleed is taken from the marginal ear vein seven days after the second boost. This test bleed (immune sera) is tested by indirect ELISA assay to determine if immune response of the rabbit is adequate for monoclonal antibody development. The best responding rabbit is given a final SQ boost and four days later is euthanized via exsanguination. The whole blood is collected via cardiac puncture. B cells producing antibody of interest are identified by indirect ELISA on target antigen and immunoglobulin genes are isolated. Heavy and light chains are cloned into separate mammalian expression vectors, transfected into HEK cells (transient transfection), and tissue culture supernatant containing rabbit monoclonal antibodies are harvested.

[00123] Example 2: Monoclonal Antibody development in mice

[00124] Female BALB/c mice (60 days old) are immunized by intraperitoneal injections (IP) with antigen/adjuvant emulsions as per standard operating procedure. Primary immunization is done with Complete Freund's Adjuvant and Incomplete Freund's Adjuvant is used for all subsequent boosts. Mice are injected IP every 3 weeks at 25µg antigen per mouse (total volume 125µL per mouse). Test bleeds are done by saphenous vein lancing 7 to 10 days after the second boost. This test bleed (immune sera) is tested by indirect ELISA assay to determine if the immune response of mice is adequate for fusion. The best 2 responding mice are given a final intravenous boost of 10µg antigen per mouse in sterile saline via lateral tail vein. 4 days after the IV boost the mice are euthanized and the spleens are harvested. Lymphocytes isolated from the spleen are used in the fusion process to produce hybridomas using the method of Kohler, G.; Milstein, C. (1975). "Continuous cultures of fused cells secreting antibody of predefined

specificity". Nature 256 (5517): 495–497. Hybridomas are generated using a PEG1500 fusion process.

[00125] Example 3: Humanization

[00126] By comparing the murine variable (V) region framework (FR) sequences of the mouse monoclonal to that of human antibodies in the Kabat data base (Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed., U.S. Department of Health and Human Services, U.S. Government Printing Office, Washington, D.C.), which is incorporated by reference, the human sequences are found to exhibit the highest degree of sequence homology to the FRs of VK and VH domains of the mouse monoclonal. Therefore, these human sequences are selected as the human frameworks onto which the CDRs for the mouse monoclonal are grafted.

[00127] Example 4. Histological Evaluations

[00128] The standard for determination of kidney injury in an animal model is careful microscopic examination of kidney morphology by a trained pathologist. See, e.g., FDA “Review of Qualification Data for Biomarkers of Nephrotoxicity Submitted by the Predictive Safety Testing Consortium, 2009, page 13 (“Histopathology was used as the gold standard that defined injury”); see also, Vaidya et al., Nat. Biotechnol. 28: 478-85, 2010 (owing to the poor sensitivity and specificity of SCr and BUN, kidney tubular damage is scored by histopathology). The Critical Path Institute’s Predictive Safety Testing Consortium (PSTC) Nephrotoxicity Working Group (NWG) created a histopathology lexicon and assigned a severity score grading scale of 0–5 to grade pathological lesions from 0 (no observable pathology), 1 (minimal), 2 (slight), 3 (moderate), 4 (marked) or 5 (severe). This grading scale was used to assess the effect of treatment with anti-TIMP-2.

[00129] Example 5:

[00130] Balb/c mice (20-24 weeks old, weight 25-30g) were purchased from Charles River Laboratories International, Inc. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh Medical Center (UPMC). After an acclimation period of 1 week, mice were subjected to a cecal ligation and puncture surgery (CLP) to induce sepsis or laparotomy only (Sham) after anesthetized with isoflurane. Specifically, the cecum was exteriorized, ligated at 1/4 from the top, and punctured through 3 times with a 21-gage needle. A droplet of feces was

extruded and the ligated and punctured cecum was replaced into the abdomen, and the abdominal wall closed. As standard post-surgical treatment, a one-time administration of fluid resuscitation (saline 40ml/kg) was performed into the scruff of the neck subcutaneously, Buprenorphine (0.05 mg/kg) injected, and antibiotics including Ceftriaxone (25mg/kg) and Metronidazole (12.5mg/kg) intraperitoneally applied immediately after surgery and every 12 hrs for 3 days. All animals were closely monitored and allowed free access to food and water during recovery. Mice were sacrificed 48 hours post the surgery and the kidney tissues harvested and blood samples obtained for further measurements.

[00131] Two isotypes of anti-TIMP2 antibodies were provided by Astute Medical, Inc. (San Diego, CA). Antibody TIMP2(NB172-77) is a monoclonal F(ab')₂, and Antibody TIMP2(NB251-47) is a polyclonal goat IgG. The CLP animals were randomized to three groups eight hours post the CLP surgery: CLP (treated with vehicle placebo), CLP+ intraperitoneal injection with anti-TIMP2 antibody NB172-77, 5mg/kg, CLP+ intraperitoneal injection with anti-TIMP2 antibody NB251-47, 5mg/kg).

[00132] Kidney injury was scored by histology in Hematoxylin and eosin (H&E) staining histology tissue images. It included cell infiltration score (0-3) and tubular epithelium injury score (0-5). Specifically, cell infiltration was determined by scoring the proximate number of cells (layers) surrounding tubular and vessel walls (score: 0 = none, 1 = <10 cells (5 layers), 2 = 10 to 20 cells (5 to 10 cell layers), 3 = > 30 cells (>10 cell layers); Injury of the tubular epithelium was scored based on the proportion of tubules that exhibit cellular necrosis, loss of brush border, cast formation, vacuolization, and tubule dilation as follows: 0, none; 1, <10%; 2, 11% to 25%; 3, 26% to 45%; 4, 46% to 75%; and 5, >76%. Serum creatinine (SCr) was measured with Creatinine Assay kit from BioVision (Mountain View, CA).

[00133] Statistic test for comparing groups was analyzed using SPSS version 14.0 software (SPSS Inc., Chicago, IL). Paired experimental groups were compared using t-test. Differences were considered significant at $p < 0.05$. Results are presented as the mean \pm SEM.

[00134] As shown in Fig. 1, anti-TIMP2 treatment with TIMP2(NB172-77) significantly decreased the extent of kidney tissue injuries compared to untreated sepsis animals (two tailed T-test, P value 0.046). As expected, anti-TIMP2 treatment has no

effect on the kidney functions measured by serum creatinine (Fig. 2). Thus, TIMP2 facilitates kidney tissue damage during sepsis independent of serum creatinine level.

[00135] While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements should be apparent without departing from the spirit and scope of the invention. The examples provided herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention.

Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

[00136] The use of “or” herein means “and/or” unless stated otherwise. Similarly, “comprise,” “comprises,” “comprising” “include,” “includes,” and “including” are interchangeable and not intended to be limiting.

[00137] It is to be further understood that where descriptions of various embodiments use the term “comprising,” those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language “consisting essentially of” or “consisting of.”

[00138] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although any methods and reagents similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods and materials are now described.

[00139] All publications mentioned herein are incorporated herein by reference in full for the purpose of describing and disclosing the methodologies, which are described in the publications, which might be used in connection with the description herein. All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains prior to the filing date of the disclosure. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure.

[00140] It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[00141] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising”, “consisting essentially of” and “consisting of” may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

[00142] Other embodiments are set forth within the following claims.

WHAT IS CLAIMED IS:

1. A method of improving kidney function in a subject in need thereof comprising administering to the subject an antibody that specifically binds Metalloproteinase inhibitor 2 (TIMP-2), optionally in association with one or more further therapeutic agents or therapeutic procedures indicated for the improvement of kidney function, in an amount sufficient to improve kidney function.
2. The method of claim 1, where the subject has chronic kidney disease (CKD) or exhibits one or more symptoms of CKD.
3. The method of claim 1, where the subject has acute kidney injury (AKI) or exhibits one or more symptoms of AKI.
4. The method of claim 1, where the subject has an existing diagnosis of one or more of congestive heart failure, preeclampsia, eclampsia, diabetes mellitus, hypertension, coronary artery disease, proteinuria, renal insufficiency, glomerular filtration below the normal range, cirrhosis, serum creatinine above the normal range, sepsis, injury to renal function, reduced renal function, or acute renal failure (ARF).
5. The method of claim 1, where the subject is undergoing or has undergone major vascular surgery, coronary artery bypass, or other cardiac surgery, and/or has received one or more of NSAIDs, cyclosporines, tacrolimus, aminoglycosides, foscarnet, ethylene glycol, hemoglobin, myoglobin, ifosfamide, heavy metals, methotrexate, radiopaque contrast agents, or streptozotocin.
6. The method of one of claims 1-5, where the subject is characterized as at or below AKIN stage 1.
7. The method of one of claims 1-5, where the subject is characterized as at or below AKIN stage 2.
8. The method of one of claims 1-5, where the subject is characterized as at or below AKIN stage 3.
9. The method of one of claims 1-8, where the subject has diabetic nephropathy (DN) or exhibits one or more symptoms of DN.

10. The method of one of claims 1-9, where the administering results in an improvement in estimated glomerular filtration rate (eGFR) of the subject.
11. The method of one of claims 1-9, where the administering reduces the level of serum creatinine in the subject.
12. The method of one of claims 1-9, where the subject is characterized as at increased risk of imminent AKI by a biomarker result.
13. The method of claim 12, wherein the biomarker result comprises one or more of a measured urinary TIMP-2 concentration and a measured urinary Insulin-like growth factor-binding protein 7 (IGFBP7) concentration.
14. The method of one of claims 1-13, wherein the subject is a human.
15. The method of one of claims 1-14, wherein the antibody that specifically binds TIMP-2 is administered parenterally.
16. The method of claim 15, wherein the antibody that specifically binds TIMP-2 is administered intravenously.
17. The method of claim 15, wherein the antibody that specifically binds TIMP-2 is administered intraarterially.
18. The method of claim 15, wherein the antibody that specifically binds TIMP-2 is administered subcutaneously.
19. The method of claim 15, wherein the antibody that specifically binds TIMP-2 is administered intraperitoneally.
20. The method of one of claims 1-19, wherein the antibody that specifically binds TIMP-2 is an IgG.
21. The method of one of claims 1-19, wherein the antibody that specifically binds TIMP-2 is an Fab fragment, an F(ab')₂, or an scFv.

22. The method of one of claims 1-21, wherein the one or more further therapeutic agents or therapeutic procedures indicated for the improvement of kidney function comprise one or more treatments selected from the group consisting of renal replacement therapy, management of fluid overload, administration of a caspase inhibitor, administration of minocycline, administration of a Poly ADP-ribose polymerase inhibitor, administration of an iron chelator, administration of a treatment for sepsis in a subject in need thereof, administration of insulin, administration of erythropoietin, and administration of a vasodilator.

FIG. 1

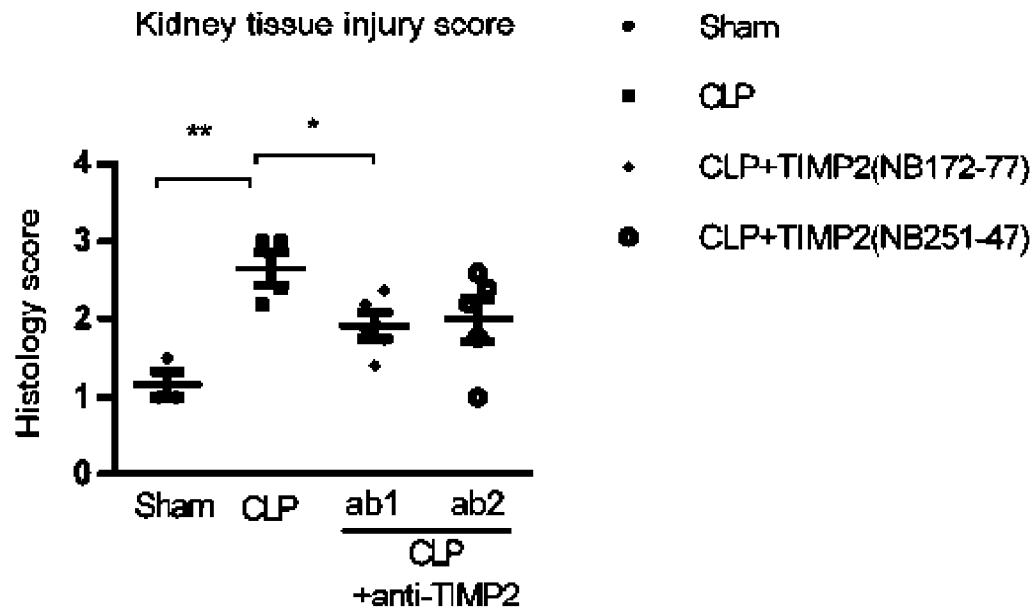
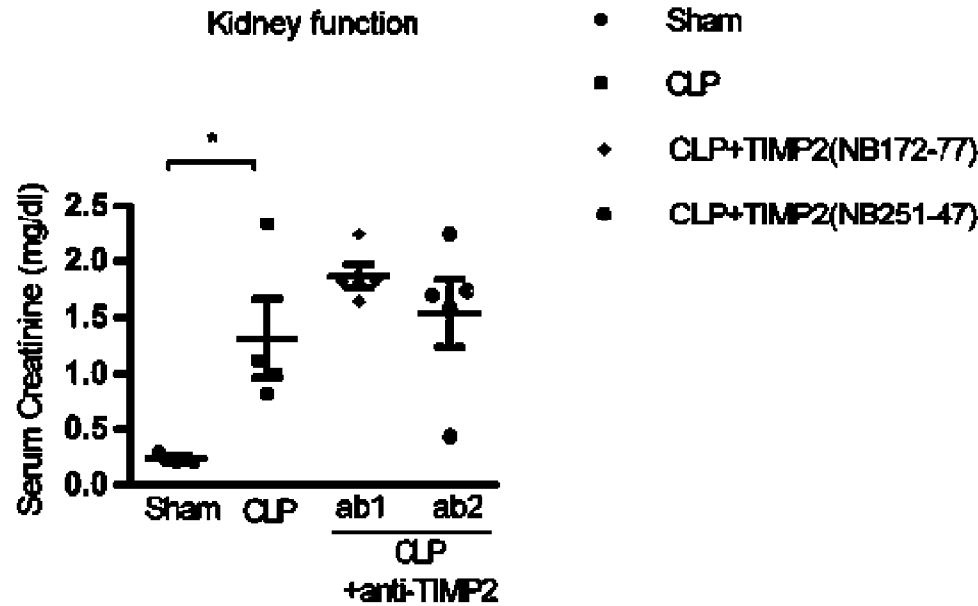


FIG. 2



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/58787

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395; A61K 39/00; A61K 38/00; G01N 33/00; C12M 3/00 (2018.01)

CPC - A61K 2039/505; C07K 2316/96; A61K 38/00; C07K 16/18; G01N 33/6893; G01N 33/54306

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)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0281083 A1 (NITTO DENKO CORPORATION) 29 September 2016 (29.09.2016), Abstract, para [0009], [0128], [0147], [0268], [0277], [0280], [0291], [0297], [0300], [0301], [0302]-[0306], [0308], [0312], [0315], [0332], [0352], and [0377]	1-8
Y	US 2016/0297893 A1 (ASTUTE MEDICAL, INC.) 13 October 2016 (13.10.2016), Abstract, para [0003], [0007], [0017], [0020], [0028], [0060], [0075], and [0081]	1-8
Y	LOPES et al., The RIFLE and AKIN classifications for acute kidney injury: a critical and comprehensive review. Clin Kidney J. 2013, Vol. 6(1), p. 8-14. Abstract; pg 8, col 1, para 1; and pg 11, col 2, and Table 3	6-8
A	AMAR et al., Potential clinical implications of recent matrix metalloproteinase inhibitor design strategies. Expert Rev Proteomics. 2015, Vol. 12(5), p. 445-7. Abstract; and pg 446, col 1, last para	1-8
A	US 2015/0355196 A1 (ASTUTE MEDICAL, INC.) 10 December 2015 (10.12.2015), Abstract, para [0004], [0017], [0028], [0051], [0080]	1-8
A	SHIBA et al., Chronic kidney disease and heart failure - Bidirectional close link and common therapeutic goal. J Cardiol. 2011, Vol. 57(1), p. 8-17. Summary; pg 10, Fig 1; and pg 12, col 1, para 2	1-8
A	YAN et al., Expression of MMP-2 and TIMP-1 in Renal Tissue of Patients with Chronic Active Antibody-mediated Renal Graft Rejection. Diagn Pathol. 2012, Vol. 7:141. PDF File: pg 1-6. Entire documentation, especially Abstract	1-8

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

<ul style="list-style-type: none"> • Special categories of cited documents: 	<ul style="list-style-type: none"> "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
<ul style="list-style-type: none"> "A" document defining the general state of the art which is not considered to be of particular relevance 	<ul style="list-style-type: none"> "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
<ul style="list-style-type: none"> "E" earlier application or patent but published on or after the international filing date 	<ul style="list-style-type: none"> "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
<ul style="list-style-type: none"> "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) 	<ul style="list-style-type: none"> "&" document member of the same patent family
<ul style="list-style-type: none"> "O" document referring to an oral disclosure, use, exhibition or other means 	
<ul style="list-style-type: none"> "P" document published prior to the international filing date but later than the priority date claimed 	

Date of the actual completion of the international search

12 January 2018

Date of mailing of the international search report

06 FEB 2018

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/58787

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OSTERMANN et al., Acute kidney injury 2016: diagnosis and diagnostic workup. Crit Care. 2016 Sep, Vol. 20(1):299. PDF File: pg 1-13. Entire documentation, especially Abstract; pg 1, col 2, para 1; and pg 2, Table 1	1-8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/58787

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 9-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.