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(54) Title: HEMICHANNEL EXTRACELLULAR-DOMAIN SPECIFIC AGENTS FOR TREATING SEPSIS

(57) Abstract: Methods of treating sepsis or endotoxemia in a subject comprising administering to the subject an amount of an antagonist of a Panxl hemichannel protein or an amount of an antagonist of a Cx43 hemichannel protein.

# HEMICHANNEL EXTRACELLULAR-DOMAIN SPECIFIC AGENTS FOR TREATING SEPSIS

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims benefit of U.S. Provisional Application No. 62/201,759, filed August 6, 2015, the contents of which are hereby incorporated by reference.

## BACKGROUND OF THE INVENTION

**[0002]** Throughout this application various patents and other publications are referred to by number in parenthesis. Full citations for the references may be found at the end of the specification. The disclosures of these references and all patents, patent application publications and books referred to herein are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

**[0003]** Bacterial infection and sepsis are the most common causes of death in the intensive care unit, annually claiming >225,000 victims in the U.S. alone. The pathogenesis of sepsis remains poorly understood, but is attributable to dysregulated systemic inflammation propagated by innate immune cells (IMCs) in response to microbial infections (1,2) and is partly attributable to dysregulated inflammatory responses sustained by proinflammatory mediators (e.g., HMGB1, CIRP, and NO). The seminal discovery of HMGB1 as a late mediator of lethal systemic inflammation (LSI) (Wang et al., Science, 285: 248-51, 1999) has prompted an investigation of the intricate mechanisms underlying the pharmacological modulation of HMGB1 secretion.

**[0004]** The present invention addresses the need for improved pharmacological treatment of sepsis, including by modulation of HMGB1 secretion via hemichannel activity.

## SUMMARY OF THE INVENTION

**[0005]** A method is provided of treating sepsis or endotoxemia in a subject or of reducing or inhibiting development of sepsis in a subject, the method comprising administering to the subject an amount of an antagonist of a Panx1 hemichannel protein or

an amount of an antagonist of a Cx43 hemichannel protein sufficient to treat sepsis or endotoxemia, or sufficient to reduce or inhibit development of sepsis.

**[0006]** Also provided is a method of treating sepsis or endotoxemia in a subject or of reducing or inhibiting development of sepsis in a subject, the method comprising administering to the subject an amount of an inhibitor of a Panx1 hemichannel protein expression or an amount of an inhibitor of a Cx43 hemichannel protein expression sufficient to treat sepsis or endotoxemia, or sufficient to reduce or inhibit development of sepsis.

**[0007]** A method of treating ischemia-reperfusion injury in a subject or of reducing or inhibiting development of an ischemia-reperfusion injury in a subject, the method comprising administering to the subject an amount of an antagonist of a Cx43 hemichannel protein sufficient to treat ischemia-reperfusion injury in a subject, or reduce or inhibit development of ischemia-reperfusion injury, in a subject.

**[0008]** Additional objects of the invention will be apparent from the description which follows.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0009]** Fig. 1. Chemical structures of a major Gancao component and derivatives. The major Gancao component, glycyrrihizic acid (GZA), can be hydrolyzed into glycyrrhitinic acid (GTA, or enoxolone) by glycaronidase *in vivo*. *In vitro*, GTA can be esterified into a succinate ester termed "carbenoxolone" (CBX).

**[0010]** Fig. 2. CBX effectively suppressed LPS-induced HMGB1 secretion and NO production. Primary peritoneal macrophages were stimulated with crude LPS in the absence or presence of CBX for 16 h, and extracellular levels of HMGB1, NO, and TNF were determined by Western blotting, Griess reaction, and ELISA, respectively. AU, arbitrary units. *, $P < 0.05$ versus "+LPS alone."

**[0011]** Fig. 3A-3B. CBX and oATP (a P2X7R antagonist) attenuated crude LPS-induced PKR phosphorylation. Primary peritoneal macrophages were stimulated with crude LPS in the absence or presence of CBX (Panel 3A) or oATP (Panel 3B) for 16 h, and cellular levels of total and phosphorylated PKR ("P-PKR") were determined by Western blotting analysis with reference to a house-keeping protein, ß-actin.

*, $P < 0.05$ vs. "- LPS - CBX"

#, $P < 0.05$ vs. "+ LPS alone."

**[0012]** Fig. 4. Delayed administration of CBX rescued mice from lethal sepsis. Balb/C mice were subjected to lethal sepsis (induced by CLP), and intraperitoneally administered

with saline or CBX at indicated doses at +24, +48 and +72 h post CLP. Animal survival rates were monitored, and the Kaplan-Meier method was used to compare the differences between groups. Arrows indicate time points of drug administration. *, $P < 0.05$ versus saline control group.

**[0013]** Fig. 5A-5B. LPS and SAA induce Panx1 expression in murine macrophages and human monocytes. Primary murine peritoneal macrophages (Panel 5A) and human peripheral blood mononuclear cells (HuPBMCs, Panel 5B) were stimulated with crude LPS or SAA for 6 h, and the cellular Panx1 levels were evaluated by immunocytochemistry (Panel 5A) and Western blotting analysis (Panel 5B), respectively.

**[0014]** Fig. 6A-6B. Cx43-and Panx1-specific peptide antagonists divergently affect septic lethality. 6A) Balb/C mice were subjected to CLP-induced sepsis, and intro peritoneally administered with saline, or Cx43- or Panx1-specifi mimetic peptide (Gap26 or 10Panx; 120 mg/kg) at +6, +18 and +36 h post CLP. Animal survival rates were monitored for two weeks, and the Kaplan-Meier method was used to compare the differences between groups. Shown was a summary of two independent experiments with similar results. *, $P < 0.05$ versus saline group. 6B) Membrane topology of Cx43 to indicate the relative localization of three mimetic peptide antagonists: Gap19, Gap26, and Gap27.

**[0015]** Fig. 7. Synthesis of small Cx43 peptide antagonists. Peptides were synthesized (ten peptides as shown in the bottom panel) corresponding to the extracellular loop 1 (EL1) of connexin 43 (Cx43). These peptides were screened for their activities in inhibiting macrophage hemichannel activities and protective efficacy in animal models of infection - or injury-elicited inflammatory diseases. (P1 is SEQ ID NO:3; P2 is SEQ ID NO:4; P3 is SEQ ID NO:5; P4 is SEQ ID NO:6; P5 is SEQ ID NO:1; P6 is SEQ ID NO:2; P7 is SEQ ID NO:7; P8 is SEQ ID NO:8; P9 is SEQ ID NO:9; and P10 is SEQ ID NO:10). The CX43 sequence shown in the top panel is SEQ ID NO:11. The CX43 extracellular loop 1 is SEQ ID NO:12. The portion of CX43 extracellular loop 1 having the sequence known as Gap26 (SEQ ID NO:13) is residues 22 through 34 of SEQ ID NO:12.

**[0016]** Fig. 8. P5 peptide (SEQ ID NO:1) significantly inhibited bacterial endotoxin-induced dye uptake. The Lucifer Yellow dye uptake was used to measure the connexin 43-gated hemichannel activities. Briefly, RAW 264.7 cells were stimulated with LPS in the absence or presence of CBX or other Cx43 peptide antagonists (GAP26 (SEQ ID NO:13) or P5) for 16 h. Subsequently, cell cultures were incubated with Lucifer Yellow (LY, 1 mg/ml) for 15 min, and fixed with 2% paraformaldehyde following three extensive washes

with 1 × PBS. The number of cells with diffused fluorescent signals was counted under a fluorescence microscope. The cells containing punctuate fluorescent signals were excluded, as the punctuate signals likely resulted from phagocytosis (rather than passive diffusion through Cx43 hemichannels) of the LY dye.

**[0017]** Fig. 9. P5 peptide significantly attenuated lethal sepsis. Balb/C mice (male, 20-25 g, 7-10 weeks) were subjected to lethal sepsis by CLP, and intraperitoneally administered with control saline (0.2 ml/mouse) or indicated peptide (10.0 mg/kg) at +0.5, +24 hours post CLP. Animal survival was assessed for up to two weeks, and the Kaplan-Meier method was used to compare the differences in mortality rates between groups. A *P* value < 0.05 was considered statistically significant. Note that peptide #2, #3, #4, #8, #9, and #10 did not confer protection against lethal sepsis. However, P5 (SEQ ID NO:1) dramatically and significantly increased animal survival. To a lesser extent, P6 (SEQ ID NO:2) also exhibited protective effect in animal models of lethal sepsis (data not shown).

**[0018]** Fig. 10. Intravenous administration of Cx43 peptide antagonist conferred protection against hepatic ischemia/reperfusion (I/R) injury. Male C57BL/6 mice (20 -25 g) were subjected to hepatic ischemia/reperfusion by temporally clamping the hepatic artery and portal vein for 60 minutes, which typically produced ischemia in 70% of the liver. At the beginning of the reperfusion, 0.2 ml saline, P5 (ENVCYD; 10.0 mg/kg BW) or P5A (ENVSYD (SEQ ID NO:14); 10.0 mg/kg BW) was injected via the internal jugular vein. At 24 h after the onset of ischemia, animals were euthanized to harvest blood to measure serum levels of hepatic injury markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercial kits. Note that P5 peptide promoted significant protection against I/R injury. *, $P < 0.05$ versus sham control; #, $P < 0.05$ versus Saline group ("I/R").

## DETAILED DESCRIPTION OF THE INVENTION

**[0019]** A method is provided of treating sepsis or endotoxemia in a subject or of reducing or inhibiting development of sepsis in a subject, the method comprising administering to the subject an amount of an antagonist of a Panx1 hemichannel protein or an amount of an antagonist of a Cx43 hemichannel protein sufficient to treat sepsis or endotoxemia, or sufficient to reduce or inhibit development of sepsis.

**[0020]** In an embodiment, the amount of the antagonist of a Panx1 hemichannel protein is administered.

**[0021]** In an embodiment, the amount of the antagonist of a Cx43 hemichannel protein is administered.

**[0022]** Also provided is a method of treating sepsis or endotoxemia in a subject or of reducing or inhibiting development of sepsis in a subject, the method comprising administering to the subject an amount of an inhibitor of a Panx1 hemichannel protein expression or an amount of an inhibitor of a Cx43 hemichannel protein expression sufficient to treat sepsis or endotoxemia, or sufficient to reduce or inhibit development of sepsis.

**[0023]** In an embodiment, the amount of the inhibitor of a Panx1 hemichannel protein expression is administered. In an embodiment, the amount of the inhibitor of a Cx43 hemichannel protein expression is administered.

**[0024]** The subject of the method may already have sepsis and the method is to treat sepsis in a subject.

**[0025]** In an embodiment, the method is to reduce or inhibit development of sepsis in a subject.

**[0026]** The subject of the method may already have endotoxemia and the method is to treat endotoxemia in a subject.

**[0027]** A method of treating ischemia-reperfusion injury in a subject or of reducing or inhibiting development of an ischemia-reperfusion injury in a subject, the method comprising administering to the subject an amount of an antagonist of a Cx43 hemichannel protein sufficient to treat ischemia-reperfusion injury in a subject, or reduce or inhibit development of ischemia-reperfusion injury, in a subject. In an embodiment, the antagonist of a Cx43 hemichannel protein is a peptide antagonist. In an embodiment, the peptide antagonist is a peptide having a sequence of an extracellular domain of a Cx43 hemichannel. In an embodiment, the antagonist of a Cx43 hemichannel protein comprises a peptide having the sequence ENVCYD (SEQ ID NO:1) or NVCYDK (SEQ ID NO:2). In an embodiment, the peptide antagonist is overlapping with a protective peptide antagonist which is Gap26 or Gap27 or Gap19. In an embodiment, the peptide antagonist is non-overlapping with a protective peptide antagonist which is Gap26 or Gap27 or Gap19. In an embodiment, the ischemia-reperfusion injury is a hepatic ischemia-reperfusion injury.

**[0028]** In an embodiment of the methods, the antagonist carbenoxolone, glycyrrhizic acid, glycyrrhitinic acid, or monoammonium glycyrrhizinate is administered. In an embodiment, the carbenoxolone, glycyrrhizic acid, glycyrrhitinic acid or monoammonium glycyrrhizinate is free of plant materials. In an embodiment, the carbenoxolone, glycyrrhizic

acid, glycyrrhitinic acid or monoammonium glycyrrhizinate is synthetically produced carbenoxolone, glycyrrhizic acid, glycyrrhitinic acid, or monoammonium glycyrrhizinate, respectively.

**[0029]** In an embodiment of the methods, the antagonist of a Cx43 hemichannel protein is a peptide antagonist. In an embodiment, the peptide antagonist is a peptide having the sequence of an extracellular domain of a Cx43 hemichannel. In an embodiment, the peptide antagonist is overlapping with a protective peptide antagonist which is Gap26 or Gap27. In an embodiment, the peptide antagonist is non-overlapping with a protective peptide antagonist which is Gap26 or Gap27 or Gap19.

**[0030]** In an embodiment of the methods, the antagonist of a Panx1 hemichannel protein can be a peptide antagonist. In an embodiment of the methods, the antagonist of a Panx1 hemichannel protein is an anti-Panx1 monoclonal antibody. In an embodiment of the methods, the antagonist of a Cx43 hemichannel protein is an anti-Cx43 monoclonal antibody.

**[0031]** In an embodiment of the methods, the antagonist of a Panx1 hemichannel protein is a small organic molecule of 1500 Da or less. In an embodiment of the methods, the antagonist of a Cx43 hemichannel protein is a small organic molecule of 1500 Da or less.

**[0032]** In an embodiment of the methods, the antagonist of a Cx43 hemichannel protein is not a gap junction antagonist.

**[0033]** In an embodiment, the inhibitor of a Panx1 hemichannel protein expression is an anti-Panx1 siRNA. In an embodiment, the inhibitor of a Cx43 hemichannel protein expression is an anti-Cx43 siRNA.

**[0034]** Antagonists of the invention can target both types of hemichannel or target one over the other. For example, the small molecule probenecid has been suggested to be a more specific inhibitor for Panx1 (over Cx43) (99); whereas another mimetic peptide, Gap19, corresponding to the intracellular loop (IL1) of Cx43 (Fig. 6B), specifically inhibits Cx43 hemichannel activities (as manifested by ATP release or dye uptake) without affecting Cx43 gap junction communication or Panx1 channel activity (100). Each of these is encompassed by the invention.

**[0035]** In general, the amount of an agent "effective" (e.g., a therapeutic agent, composition, and/or formulation) is an amount effective to achieve a stated effect, to elicit the desired biological response. In some embodiments, a therapeutically effective amount of

a substance is an amount that is sufficient, when administered to a subject suffering from or susceptible to a disease, disorder, and/or condition, to treat, diagnose, prevent, and/or delay the onset of one or more symptoms of the disease, disorder, and/or condition. As will be appreciated by those of ordinary skill in this art, and effective amount of a substance may vary depending on such factors as the desired biological endpoint, the substance to be delivered, the pharmacokinetics of the compound, the target cell or tissue, the disease being treated, the mode of administration, and the patient, etc. For example, the effective amount of a composition and/or formulation to treat a disease, disorder, and/or condition is the amount that alleviates, ameliorates, relieves, inhibits, prevents, delays onset of, reduces severity of and/or reduces incidence of one or more symptoms or features of the disease, disorder, and/or condition. Those of ordinary skill in the art will appreciate that, commonly, an effective amount will be administered over a series of individual doses. In some embodiments, the term "effective amount" when used in a pharmaceutical context (e.g., pharmaceutically effective amount) means that an agent is present in an amount sufficient to achieve a desired therapeutic effect.

**[0036]** Routes of administration encompassed by the methods of the invention include, but are not limited to, each of the following individual routes, and any subset thereof, auricular, buccal, conjunctival, cutaneous, subcutaneous, endocervical, endosinusial, endotracheal, enteral, epidural, via hemodialysis, interstitial, intrabdominal, intraamniotic, intra-arterial, intra-articular, intrabiliary, intrabronchial, intrabursal, intracardiac, intracartilaginous, intracaudal, intracavernous, intracavitary, intracerebral, intracisternal, intracorneal, intracoronary, intradermal, intradiscal, intraductal, intraepidermal, intraesophagus, intragastric, intravaginal, intragingival, intraileal, intraluminal, intralesional, intralymphatic, intramedullary, intrameningeal, intramuscular, intraocular, intraovarian, intraepicardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrasinal, intraspinal, intrasynovial, intratendinous, intratesticular, intrathecal, intrathoracic, intratubular, intratumor, intratympanic, intrauterine, intravascular, intravenous, intraventricular, intravesical, intravitreal, laryngeal, nasal, nasogastric, ophthalmic, oral, oropharyngeal, parenteral, percutaneous, periarticular, peridural, rectal, inhalationally, retrobulbar, subarachnoid, subconjuctival, sublingual, submucosal, topically, transdermal, transmucosal, transplacental, transtracheal, ureteral, uretheral, and vaginal administration.

**[0037]** In an embodiment of the methods, the subject is human.

**[0038]** In an embodiment, the Cx43 is Human Cx43 having the following sequence (SEQ ID NO:15):

MGDWSALGKLLDKVQAYSTAGGKVWLSVLFIFRILLLGTAVESAWGDEQSAFRCN
TQQPGCENVCYDKSFPISHVRFWVLQIIFVSVPTLLYLAHVFYVMRKEEKLNKKEE
ELKVAQTDGVNVDMHLKQIEIKKFKYGIEEHGKVKMRGGLLRTYIISILFKSIFEVA
FLLIQWYIYGFSLSAVYTCKRDPCPHQVDCFLSRPTEKTIFIIFMLVVSLVSLALNIIEL
FYVFFKGVKDRVKGKSDPYHATSGALSPAKDCGSQKYAYFNGCSSPTAPLSPMSPP
GYKLVTGDRNNSSCRNYNKQASEQNWANYSAEQNRMGQAGSTISNSHAQPFDFP
DDNQNSKKLAAGHELQPLAIVDQRPSSRASSRASSRPRPDDLEI.

**[0039]** In an embodiment, the Panx1 is Human Panx1 having the following sequence (SEQ ID NO:16):

MAIAQLATEYVFSDFLLKEPTEPKFKGLRLELAVDKMVTCIAVGLPLLLISLAFAQEI
SIGTQISCFSPSSFSWRQAAFVDSYCWAAVQQKNSLQSESGNLPLWLHKFFPYILLL
FAILLYLPPLFWRFAAAPHICSDLKFIMEELDKVYNRAIKAAKSARDLDMRDGACS
VPGVTENLGQSLWEVSESHFKYPIVEQYLKTKKNSNNLIIKYISCRLLTLIIILLACIY
LGYYFSLSSLSDEFVCSIKSGILRNDSTVPDQFQCKLIAVGIFQLLSVINLVVYVLLAP
VVVYTLFVPFRQKTDVLKVYEILPTFDVLHFKSEGYNDLSLYNLFLEENISEVKSYK
CLKVLENIKSSGQGIDPMLLLTNLGMIKMDVVDGKTPMSAEMREEQGNQTAELQG
MNIDSETKANNGEKNARQRLLDSSC.

**[0040]** In an embodiment, the Gap26 has the sequence: VCYDKSFPISHVR (SEQ ID NO:17).

**[0041]** In an embodiment, the Gap27 has the sequence: SRPTEKTIFII (SEQ ID NO:18).

**[0042]** In an embodiment, the Gap19 has the sequence: KQIEIKKFK (SEQ ID NO:19).

**[0043]** All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

**[0044]** This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

## Experimental Results

*Introduction*

**[0045]** It was recently shown that ultrapure lipopolysaccharide (LPS) (10 μg/ml) can stimulate macrophages to produce early cytokines (e.g., TNF), but completely fails to trigger HMGB1 secretion unless the initial LPS priming is accompanied by a second stimulus (e.g., ATP) (43,44). Similarly, ATP itself is unable to induce HMGB1 secretion without prior LPS exposure (43), even though it can facilitate PKR phosphorylation (44) and inflammasome activation (46-48). In contrast, prolonged stimulation with the commonly-used (and presumably also more clinically relevant) crude LPS (containing <1% bacterial proteins and nucleic acids such as CpG-DNA) led to dramatic PKR phosphorylation (49) and HMGB1 secretion (21).

**[0046]** Macrophages/monocytes release ATP through the membrane hemichannels composed of connexins (Cx) or pannexins (Panx). Despite of the lack of amino acid sequence homology, Cx43 and Panx1 exhibit similar membrane topology by carrying four transmembrane domains, one intracellular loop (along with the N and C termini), and two extracellular loops. Although both can oligomerize to form the hexameric half channel (or "hemichannel") only Cx43 hemichannels can dock with the hemichannels on adjacent cells to form gap junction channels (GJC) that facilitate intercellular communication in the heart, vasculature, and brain. Nevertheless, both Cx43 and Panx1 hemichannels may provide a temporal mode of ATP release from activated innate immune cells (e.g., monocytes, macrophages and neutrophils) (51,52). For instance, Panx1 has been shown to contribute to ATP release from apoptotic cells (53,54), because pharmacological inhibition (by a Gancao component derivative, CBX, >100 μM) or genetic knock-down of PANX1 uniformly attenuated ATP liberation. It has been suggested that ATP contributes to inflammasome activation through activating the purinergic P2X7 receptor (P2X7R) (50). The activation of P2X7R results in an immediate (within milliseconds) opening of ATP-gated P2X7R channel permeable for small cations ($Ca^{2+}$), followed by a gradual recruitment and opening (over seconds to minutes) of larger Panx1 hemichannels, allowing passage of larger anionic molecules up to 900 Da (e.g., ATP) (55-57). This Panx1-mediated feed-forward ATP release contributes to the LPS-stimulated inflammasome activation (58) and subsequent inflammasome-dependent cytokine release (46-48,59,60).

**[0047]** Gancao (*Radix glycyrrhizae*, or licorice) has been traditionally used in the treatment of peptic ulcer, hepatic injury, and hepatitis, but its protective mechanisms

remain elusive. Data disclosed herein indicates that carbenoxolone (CBX), a derivative of the major Gancao component, glycyrrhizin (glycyrrhizic acid, GZA), dose-dependently abrogated LPS-induced PKR (dsRNA-activated protein kinase R) phosphorylation and HMGB1 secretion, and rescues mice from lethal sepsis (induced by cecal ligation and puncture, CLP) even if given in a delayed fashion.

*Examples*

**[0048]** Multiple herbal components were screened for activities in inhibiting LPS-induced PKR activation and HMGB1 secretion. Gancao (*Radix glycyrrhizae*) has been traditionally used for many centuries in the treatment of various inflammatory ailments including peptic ulcer, hepatitis, and pulmonary bronchitis. Its anti-inflammatory properties are attributable to a major component, glycyrrhizin (glycyrrhizic acid, GZA, Fig. 1), which has been proven beneficial in animal models of hepatitis (61, hepatic ischemia/reperfusion (I/R) (62,63), toxin-induced liver injury (64,65), endotoxemia (66,67), and colitis (68). The replacement of the glucuronic acid in GZA by succinic acid gives rise to a new compound, carbenoxolone (CBX, Fig. 1), a drug previously prescribed for patients with esophageal ulceration and other inflammation ailments (69).

**[0049]** Since its inception, CBX has been shown to dose-dependently inhibit a variety of biological activities including the Cx43 gap junctions ($EC_{50}$ = 50-100 μM) and the Panx1 hemichannels ($EC_{50}$ = 1-4 μM) (70,71). For instance, it was previously shown that CBX (10 μM) effectively inhibited the Panx1 hemichannel-mediated ATP release in response to hypoxia (72), sheer stress (73), and low oxygen tension (74). Furthermore, CBX can inhibit LPS-induced dye uptake (55,75), and confer protection against LPS-induced acute lung injury (76), and cerebral ischemic injury (77). Herein it is disclosed that CBX remarkably inhibited endotoxin-induced nitric oxide production and HMGB1 secretion in macrophage cultures (Fig. 2), confirming Gancao's anti-inflammatory properties. However, it is unlikely that CBX inhibits the LPS-induced HMGB1 secretion through impairing the Cx43 gap junctions, because macrophages do not form gap junctions with themselves, and the concentrations of CBX used to block gap junctions (e.g., 50-100 μM) in other cell types are much higher than those (e.g., 5-10 μM) used to abrogate LPS-induced HMGB1 secretion in macrophages (49). It is not known, however, if CBX attenuates Cx43 hemichannel-mediated ATP release, thereby affecting subsequent PKR activation and HMGB1 secretion. To test this possibility, it was determined whether CBX affected membrane hemichannel

activities by measuring the cellular uptake of an anionic dye, Lucifer Yellow (LY, MW = 444 Da). In quiescent macrophages, approximately 2% cells displayed diffuse fluorescent signal after LY incubation, whereas prolonged LPS stimulation elevated the number of LY-positive cells to ~16%, suggesting that LPS increased macrophage hemichannel activities (49). However, CBX significantly reduced the number of LY-positive cells to 6-8%, suggesting that CBX effectively inhibits LPS-induced HMGB1 secretion by blocking macrophage hemichannel activities.

**[0050]** One of the key ATP receptors, $P2X_7R$ may be important in LPS-induced HMGB1 secretion because a specific $P2X_7R$ antagonist, oATP, similarly inhibited LPS-induced LY-uptake and HMGB1 secretion (data not shown). In light of the roles of P2X7R and PKR in LPS/ATP-induced inflammasome activation (44,58), it was tested whether CBX and P2X7R antagonists (e.g., oATP) have an effect on LPS-induced PKR activation in primary macrophage cultures. Remarkably, prolonged stimulation with crude LPS (containing trace amounts of bacterial proteins and nucleic acids) resulted in a >2-fold increase of total PKR protein levels, but a more robust (> 8-fold) elevation of phosphorylated PKR levels (Fig. 3A, 3B). Furthermore, this LPS-induced elevation of PKR expression and phosphorylation was significantly attenuated both by CBX (Fig. 3A) and oATP (Fig. 3B), suggesting an important role for hemichannels and PKR activation in LPS-induced HMGB1 secretion.

**[0051]** Given the pathogenic role of HMGB1 in lethal sepsis (32), the therapeutic potential of CBX was explored using a clinically relevant animal model of polymicrobial sepsis induced by CLP. The first dose of CBX was given 24 h after CLP, a time point at which mice developed clear signs of sepsis including lethargy, diarrhea, and piloerection. Repeated administration of CBX beginning 24 h after the onset of sepsis (followed by additional doses at 48 and 72 h post CLP) conferred a dose-dependent and significant protection (Fig. 4), supporting CBX as a therapeutic in the treatment of sepsis. Data obtained here (not shown) indicates that CBX binds to HMGB1 A-box with an affinity > 20-fold higher than that of the most well-known HMGB1 inhibitor, GZA (111).

**[0052]** It was previously unknown whether the pharmacological targets of CBX, such as Panx1 and Cx43, also occupy a pathogenic role in LSI possibly by facilitating ATP efflux, PKR activation and HMGB1 secretion. It was investigated whether prolonged stimulation with crude LPS or other HMGB1 secretion stimuli (such as SAA or CIRP) may upregulate the expression of Panx1 and/or Cx43 hemichannel, which facilitate ATP efflux, PKR

phosphorylation, and subsequent HMGB1 secretion, thereby contributing to the pathogenesis of LSI. A hypothesis was proposed that exogenous PAMPs (LPS) and endogenous proinflammatory mediators (SAA or CIRP) regulate HMGB1 secretion through stimulating Panx1 and/or Cx43 hemichannel expression and PKR phosphorylation. In light of the important role of Cx43 and Panx1 in ATP efflux, in conjunction with the requirement of ATP for ultrapure LPS-induced HMGB1 secretion, it was desirable to determine whether crude LPS and other key HMGB1 secretion stimuli (e.g., CIRP and SAA) can uniformly modulate hemichannel expression and PKR/STAT1 phosphorylation.

**[0053]** Primary murine macrophages and human monocytes were employed to investigate the mechanisms underlying the regulation of HMGB1 secretion by various inflammatory stimuli. Primary peritoneal macrophages were isolated from Balb/C mice (male, 7-8 weeks, 20-25 grams) at 2-3 days after intraperitoneal injection of 2 ml thioglycollate broth (4%, Difco, Detroit, MI) as previously described (23,78). Human peripheral blood mononuclear cells (HuPBMCs) were isolated from human blood purchased from the New York (Long Island) Blood Bank (Melville, NY) by density gradient centrifugation through Ficoll (Ficoll-Paque PLUS, Pharmacia, Piscataway, NJ) as previously described (23,78,79). At 80-90% confluence, macrophage/monocyte cultures will be stimulated with divergent stimuli: ultrapure and crude LPS, CIRP 14, SAA (PeproTech, Cat. No. 300-13) at different concentrations and for various time periods (0, 6, 12, and 18 h). The expression levels of Cx43 or Panx1 hemichannel proteins in macrophage/monocyte cultures are determined by Western blotting or immunocytochemistry techniques as previously described (21,23,80).

**[0054]** The expression of hemichannel proteins might be regulated differentially in immune versus non-immune cells. For instance, LPS down-regulates Cx43 expression in the liver and heart (81), but up-regulates it in the kidney, lung (82), and IMCs (83,84). Accordingly, prolonged stimulation with crude LPS, SAA, or CIRP will similarly upregulate Cx43 and Panx1 hemichannel proteins in monocyte/macrophage cultures. Indeed, it was found that both LPS and SAA effectively elevated cellular levels of Panx1 in both murine macrophages (Fig. 5A) and human monocytes (Fig. 5B). Consistent with the notion that the enzymatic cleavage of Panx1 by caspase 3 in apoptotic cells is required for activation and opening of Panx1 hemichannels (53), it was found that the upregulation of Panx1 was accompanied by the appearance of a smaller molecular weight (10 kDa) band (Fig. 5B), possibly indicative of a Panx1 degradation product.

**[0055]** Roles of hemichannels in the pathogenesis of LSI: The data obtained here indicated that LPS and SAA upregulated the expression of Panx1 and Cx43 in primary murine macrophages and human monocytes. This is interesting in view of a recent study that suggested conditional knockout of Cx43 in the CD11c-expressing leukocytes rendered mice more susceptible to lethal endotoxemia (105), reinforcing the notion of a beneficial role of Cx43 in alveolar macrophage-epithelium gap junction communication (GJCs). In light of the possible roles of these hemichannel proteins in ATP-dependent PKR activation and HMGB1 secretion, it was determined whether alterations of these hemichannel activities (by using mimetic peptide antagonists, neutralizing antibodies, or herbal inhibitors such as GZA) or protein levels (by gene KO) affect animal survival in endotoxemia and CLP-induced sepsis.

**[0056]** Sepsis is commonly simulated in animals by intraperitoneally administering a bolus and known amount of endotoxin (endotoxemia), or by surgically inducing peritonitis via perforating the cecum - a technique known as cecal ligation and puncture (CLP) as previously described (21,78,94,95). To understand the possible role of hemichannel proteins in LSI, male Balb/C mice (7-8 weeks, 20-25 g) are subjected to lethal endotoxemia or sepsis, and a wide range of hemichannel inhibitors (e.g., mimetic peptide antagonists, and herbal component, GZA) is injected intraperitoneally at various doses and time points (0.5, 12, and 24 h) after the onset of endotoxemia or sepsis. Their effects on the outcomes of LSI are assessed by comparing the long-term (two-week) survival rates between the anti-hemichannel-treated groups with vehicle-treated controls.

**[0057]** To modulate the hemichannel activities, various mimetic "gap" peptides have been designed to mimic the extracellular loops of Cx43 and Panx1. For instance, Gap26 and Gap27 mimic a short stretch of amino acids on the first and second extracellular loops (EL1 and EL2, Fig. 6B), and are expected to interact with the extracellular loops of the Cx43 (96), thereby inhibiting Cx43 hemichannel activities or Cx43 gap junction formation. Similarly, a Panx1-specific mimetic inhibitory peptide, 10Panx, has been shown to selectively attenuate P2X7R-induced Panx1 hemichannel activation (97,98). It was found that the Panx1-specific mimetic peptide, 10Panx, reproducibly exacerbated CLP-induced animal lethality when given repetitively at a dose of 120 mg/kg (Fig. 6A). In a sharp contrast, repetitive administration of a Cx43-specific mimetic peptide (Gap26, at + 6 and +18, and 36 h post CLP; 120 mg/kg), promoted a reproducible and significant protection against lethal sepsis (Fig. 6A).

**[0058]** To further confirm the pathogenic role of Cx43 in LSI, monoclonal antibodies (MAbs) are generated targeting extracellular loops of Cx43, and it is tested whether Cx43-specific MAbs similarly protects animals against lethal sepsis. Multiple hyridomas have been generated by this laboratory that produce Gap26-reactive antibodies, and the antibodies will be screened for their activities in inhibiting hemichannel activities using the LY dye uptake or ATP release assays (49).

**[0059]** Peptides were synthesized (ten peptides as shown in the bottom panel) corresponding to the extracellular loop 1 (EL1) of connexin 43 (Cx43). These peptides were screened for their activities in inhibiting macrophage hemichannel activities and protective efficacy in animal models of infection- or injury-elicited inflammatory diseases.

**[0060]** P5 peptide (SEQ ID NO:1) significantly inhibited bacterial endotoxin-induced dye uptake. The Lucifer Yellow dye uptake was used to measure the connexin 43-gated hemichannel activities. Briefly, RAW 264.7 cells were stimulated with LPS in the absence or presence of CBX or other Cx43 peptide antagonists (GAP26 (SEQ ID NO:13) or P5) for 16 h. Subsequently, cell cultures were incubated with Lucifer Yellow (LY, 1 mg/ml) for 15 min, and fixed with 2% paraformaldehyde following three extensive washes with 1 × PBS. The number of cells with diffused fluorescent signals was counted under a fluorescence microscope. The cells containing punctuate fluorescent signals were excluded, as the punctuate signals likely resulted from phagocytosis (rather than passive diffusion through Cx43 hemichannels) of the LY dye. P5 peptide also significantly attenuated lethal sepsis. Balb/C mice (male, 20-15 g, 7-10 weeks) were subjected to lethal sepsis by CLP, and intraperitoneally administered with control saline (0.2 ml/mouse) or indicated peptide (10.0 mg/kg) at +0.5, +24 hours post CLP. Animal survival was assessed for up to two weeks, and the Kaplan-Meier method was used to compare the differences in mortality rates between groups. A P value $< 0.05$ was considered statistically significant. Note that peptide #2, #3, #4, #8, #9, and #10 did not confer protection against lethal sepsis. However, P5 (SEQ ID NO:1) dramatically and significantly increased animal survival. To a lesser extent, P6 (SEQ ID NO:2) also exhibited protective effect in animal models of lethal sepsis (data not shown).

**[0061]** Intravenous administration of Cx43 peptide antagonist conferred protection against hepatic ischemia/reperfusion (I/R) injury. Male C57BL/6 mice (20 -25 g) were subjected to hepatic ischemia/reperfusion by temporally clamping the hepatic artery and portal vein for 60 minutes, which typically produced ischemia in 70% of the liver. At the

beginning of the reperfusion, 0.2 ml saline, P5 (ENVCYD, 10.0 mg/kg BW) or P5A (ENVSYD (SEQ ID NO:14), 10.0 mg/kg) was injected via the internal jugular vein. At 24 h after the onset of ischemia, animals were euthanized to harvest blood to measure serum levels of hepatic injury markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using commercial kits. Note that P5 peptide promoted significant protection against I/R injury. *, $P < 0.05$ versus sham control; #, $P < 0.05$ versus Saline group ("I/R").

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114. Luo,L., Jin,Y., Kim,I.D. & Lee,J.K. Glycyrrhizin Suppresses HMGB1 Inductions in the Hippocampus and Subsequent Accumulation in Serum of a Kainic Acid-Induced Seizure Mouse Model. Cell Mol. Neurobiol. 34, 987-997 (2014).

115. Musumeci,D., Roviello,G.N. & Montesarchio,D. An overview on HMGB1 inhibitors as potential therapeutic agents in HMGB1-related pathologies. Pharmacol. Ther. 141, 347-357 (2014).

116. Ohnishi,M. et al. HMGB1 inhibitor glycyrrhizin attenuates intracerebral hemorrhage-induced injury in rats. Neuropharmacology. 61, 975-980 (2011).

117. Yang,H. et al. MD-2 is required for disulfide HMGB1-dependent TLR4 signalingGOOGLE. J.Exp.Med. in press. 2014.

118. Yang,H. et al. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc. Natl. Acad. Sci. U. S. A. 107, 11942-11947 (2010).

What is claimed is:

1. A method of treating sepsis or endotoxemia in a subject or of reducing or inhibiting development of sepsis in a subject, the method comprising administering to the subject an amount of an antagonist of a Cx43 hemichannel protein or an amount of an antagonist of a Panx1 hemichannel protein sufficient to treat sepsis or endotoxemia, or sufficient to reduce or inhibit development of sepsis.

2. The method of Claim 1, wherein the amount of the antagonist of a Cx43 hemichannel protein is administered.

3. The method of Claim 1, wherein the amount of the antagonist of a Panx1 hemichannel protein is administered.

4. A method of treating sepsis or endotoxemia in a subject or of reducing or inhibiting development of sepsis in a subject, the method comprising administering to the subject an amount of an inhibitor of a Cx43 hemichannel protein expression or an amount of an inhibitor of a Panx1 hemichannel protein expression sufficient to treat sepsis or endotoxemia, or sufficient to reduce or inhibit development of sepsis.

5. The method of Claim 4, wherein the amount of the inhibitor of a Cx43 hemichannel protein expression is administered.

6. The method of Claim 4, wherein the amount of the inhibitor of a Panx1 hemichannel protein expression is administered.

7. The method of any of Claims 1-6, wherein the subject already has sepsis and the method is to treat sepsis in the subject.

8. The method of any of Claims 1-6, wherein the method is to reduce or inhibit development of sepsis in the subject.

9. The method of any of Claims 1-6, wherein the subject already has endotoxemia and the method is to treat endotoxemia in the subject.

10. The method of any of Claims 1-2 or 7-9, wherein the antagonist of a Cx43 hemichannel protein is a peptide antagonist.

11. The method of any of Claims 1, 3 or 7-9, wherein the antagonist of a Panx1 hemichannel protein is a peptide antagonist.

12. The method of Claim 11, wherein the peptide antagonist is a peptide having the sequence of an extracellular domain of a Cx43 hemichannel.

13. The method of any of Claims 1-2 or 7-9, wherein the antagonist of a Cx43 hemichannel protein comprises a peptide having the sequence ENVCYD (SEQ ID NO:1) or NVCYDK (SEQ ID NO:2).

14. The method of Claim 12, wherein the peptide antagonist is overlapping with a protective peptide antagonist which is Gap26 or Gap27.

15. The method of Claim 12, wherein the peptide antagonist is non-overlapping with a protective peptide antagonist which is Gap26 or Gap27.

16. The method of any of Claims 1-9, wherein carbenoxolone, glycyrrhizic acid, glycyrrhitinic acid, or monoammonium glycyrrhizinate is administered.

17. The method of Claim 16, wherein carbenoxolone, glycyrrhizic acid, glycyrrhitinic acid or monoammonium glycyrrhizinate is free of plant materials.

18. The method of any of Claims 16-17, wherein the carbenoxolone, glycyrrhizic acid, glycyrrhitinic acid or monoammonium glycyrrhizinate is synthetically produced carbenoxolone, glycyrrhizic acid, glycyrrhitinic acid, or monoammonium glycyrrhizinate, respectively.

19. The method of any of Claims 1-2 or 7-9, wherein the antagonist of a Panx1 hemichannel protein is an anti-Panx1 monoclonal antibody.

20. The method of any of Claims 1, 3 or 7-9, wherein the antagonist of a Cx43 hemichannel protein is an anti-Cx43 monoclonal antibody.

21. A method of treating ischemia-reperfusion injury in a subject or of reducing or inhibiting development of an ischemia-reperfusion injury in a subject, the method comprising administering to the subject an amount of an antagonist of a Cx43 hemichannel protein sufficient to treat ischemia-reperfusion injury in a subject, or reduce or inhibit development of ischemia-reperfusion injury, in a subject.

22. The method of Claim 21, wherein the antagonist of a Cx43 hemichannel protein is a peptide antagonist.

23. The method of Claim 22, wherein the peptide antagonist is a peptide having a sequence of an extracellular domain of a Cx43 hemichannel.

24. The method of any of Claims 21-23, wherein the antagonist of a Cx43 hemichannel protein comprises a peptide having the sequence ENVCYD (SEQ ID NO:1) or NVCYDK (SEQ ID NO:2).

25. The method of Claim 22, wherein the peptide antagonist is overlapping with a protective peptide antagonist which is Gap26 or Gap27.

26. The method of Claim 22, wherein the peptide antagonist is non-overlapping with a protective peptide antagonist which is Gap26 or Gap27.

27. The method of any of Claims 21-26, wherein the ischemia-reperfusion injury is a hepatic ischemia-reperfusion injury.

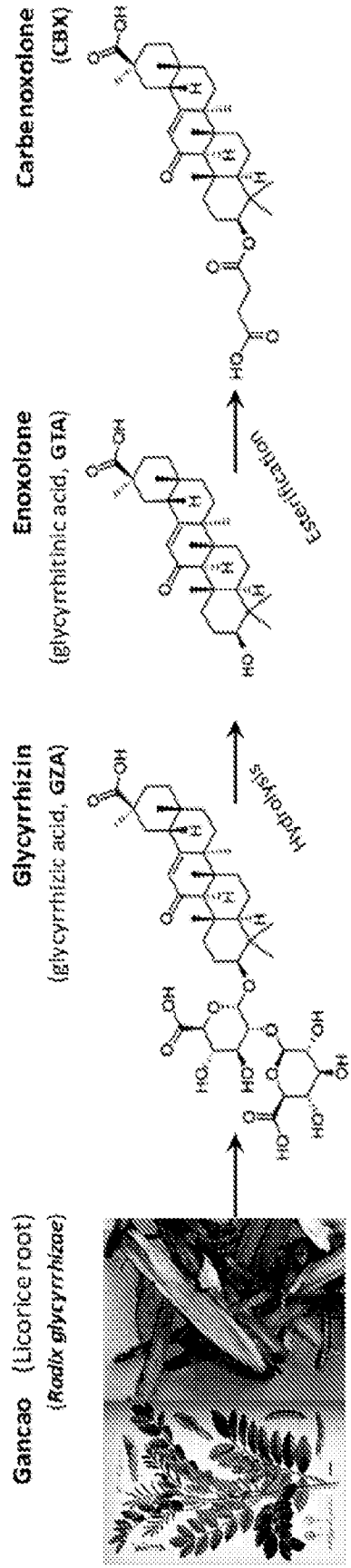

Gancao (Licorice root)
(Radix glycyrrhizae)
Glycyrrhizin
(glycyrrhizic acid, GZA)
Hydrolysis
Enoxolone
(glycyrrhitinic acid, GTA)
Esterification
Carbenoxolone
(CBX)

Fig 1

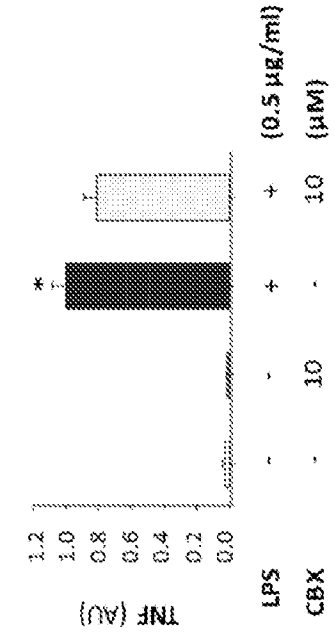



Fig 2C

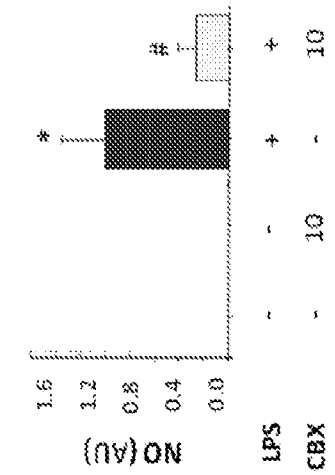



Fig 2B

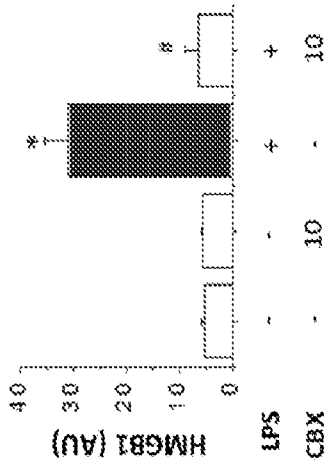



Fig 2A

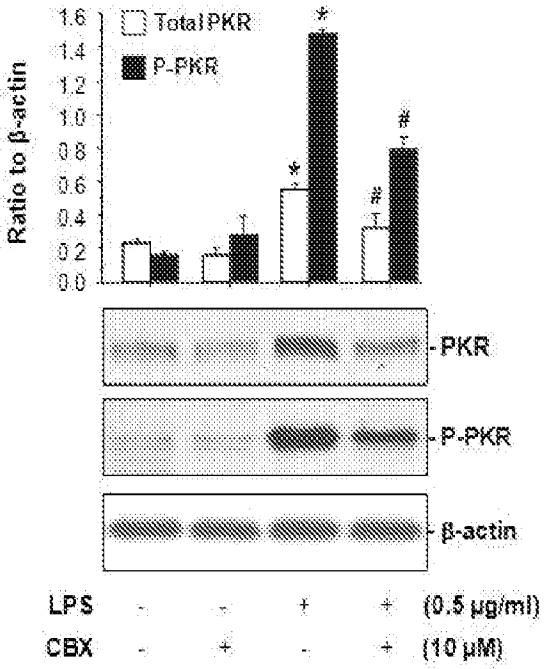

Ratio to β-actin
Total PKR
P-PKR
PKR
P-PKR
β-actin
LPS - - + + (0.5 μg/ml)
CBX - + - + (10 μM)

Fig 3A

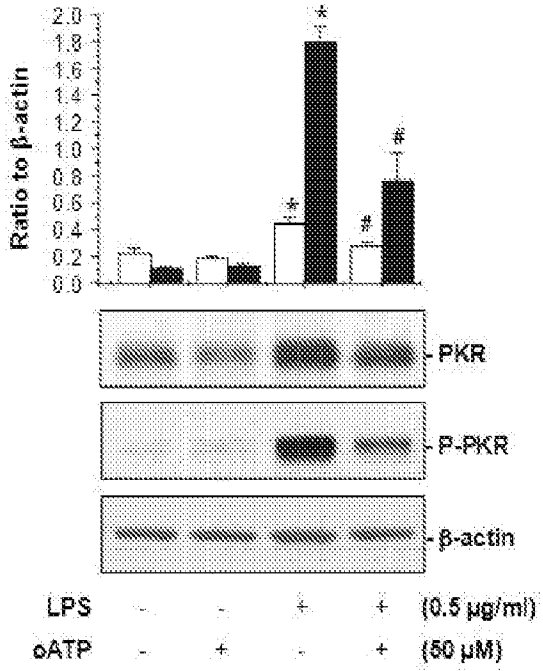

Ratio to β-actin
PKR
P-PKR
β-actin
LPS - - + + (0.5 μg/ml)
oATP - + - + (50 μM)

Fig 3B

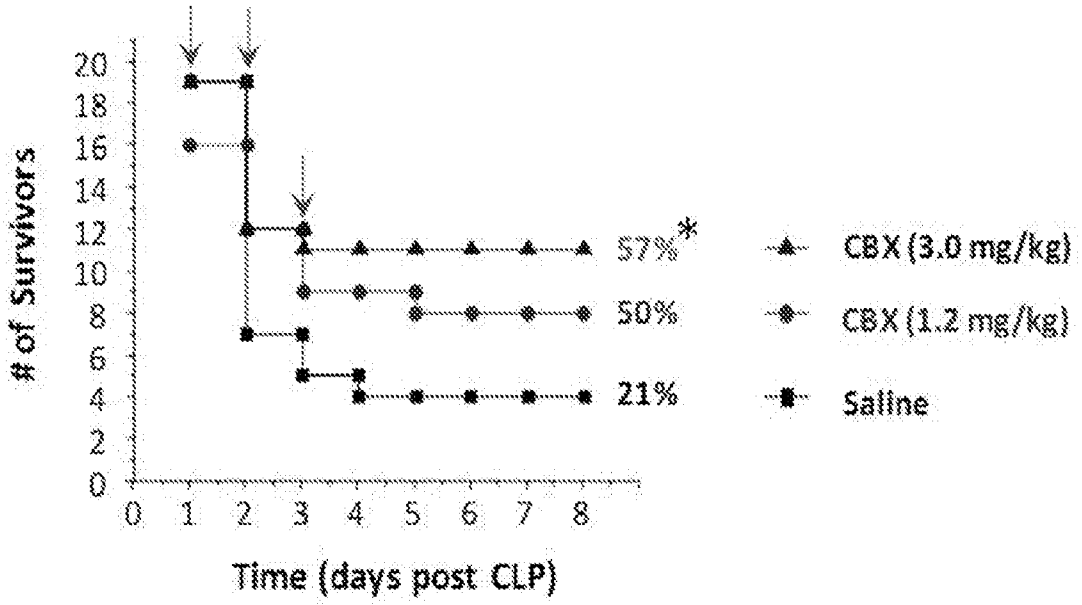

# of Survivors
20
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8
Time (days post CLP)
57%*
50%
21%
CBX (3.0 mg/kg)
CBX (1.2 mg/kg)
Saline

Fig 4

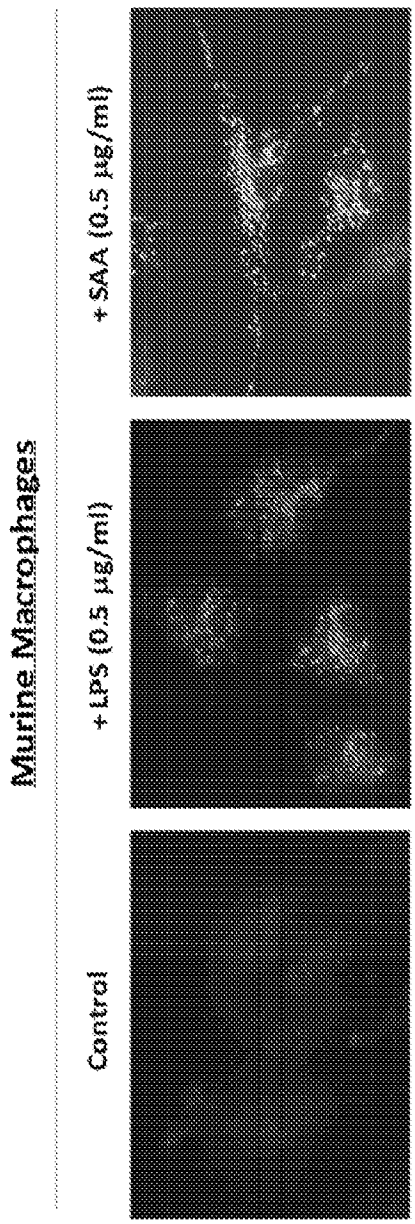

Murine Macrophages
Control
+ LPS (0.5 μg/ml)
+ SAA (0.5 μg/ml)

Fig 5A

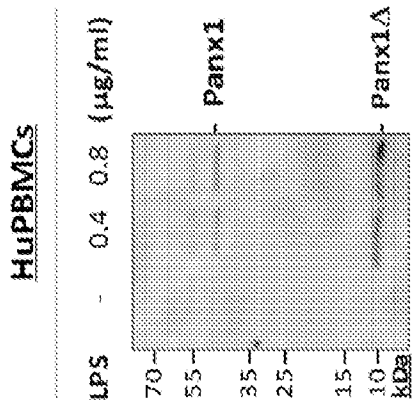

HuPBMCs
LPS - 0.4 0.8 (μg/ml)
Panx1
Panx1Δ
70
55
35
25
15
10
kDa

Fig 5B

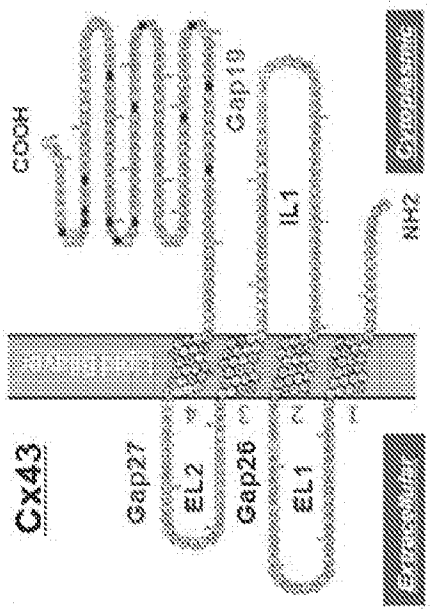

COOH
Cx43
Gap27
EL2
Gap26
EL1
Gap18
IL1
NH2

Fig 6B

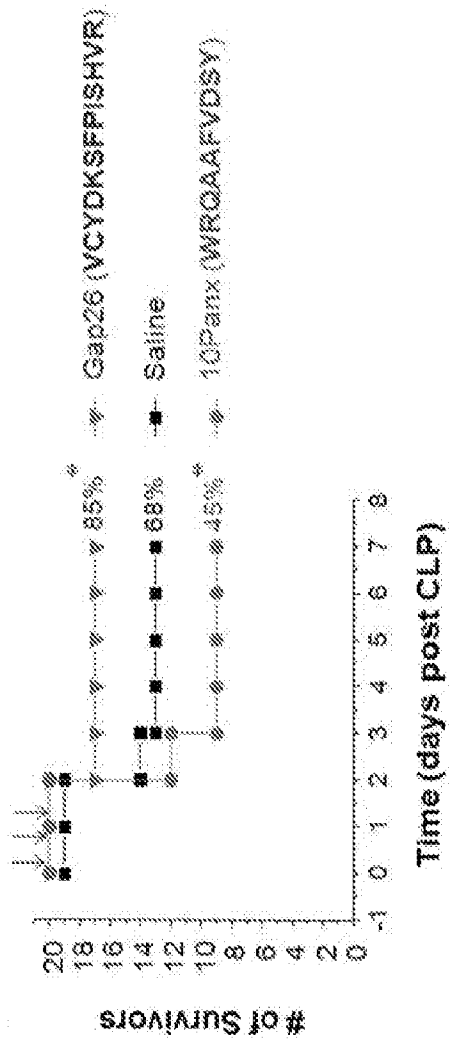
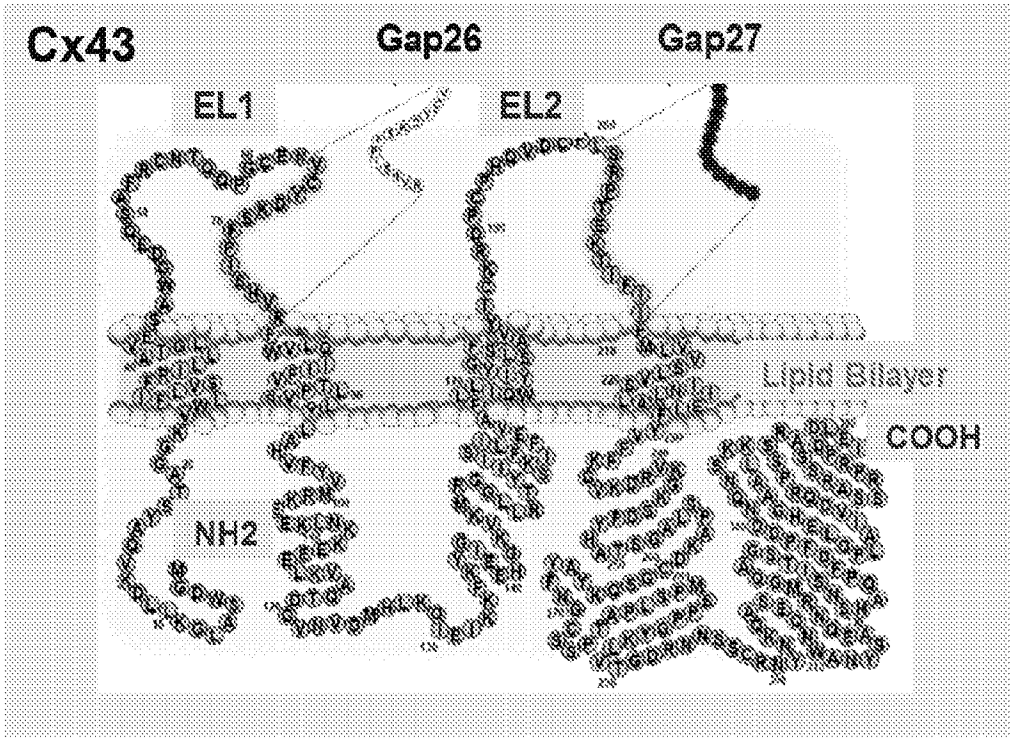

# of Survivors
Time (days post CLP)
Gap26 (VCYDKSFPISHVR)
Saline
10Panx (WRQAAFVDSY)
85%
68%
45%
*
*

Fig 6A


Cx43
Gap26
Gap27
EL1
EL2
Lipid Bilayer
COOH
NH2

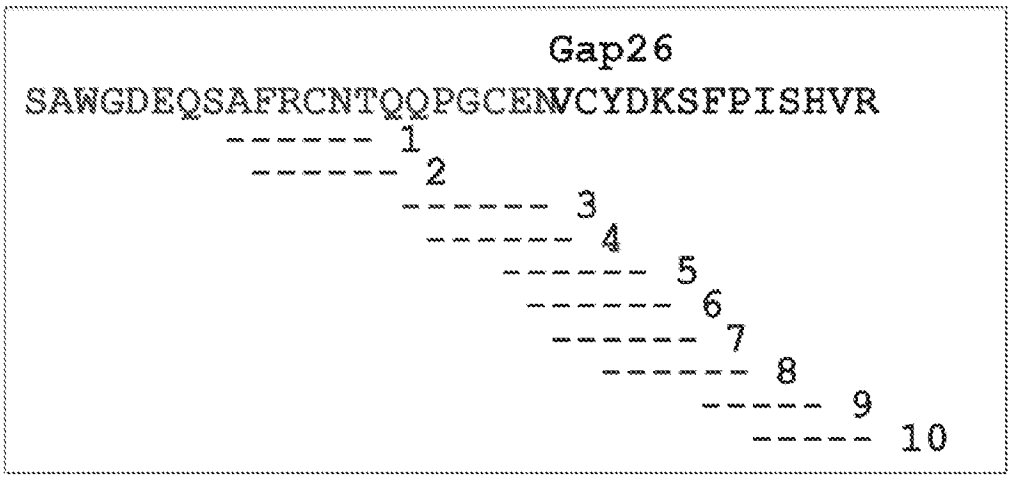

Cx43 Extracellular Loop 1 (EL1)
Gap26
SAWGDEQSAFRCNTQQPGCENVCYDKSFPISHVR
1
2
3
4
5
6
7
8
9
10

Fig 7

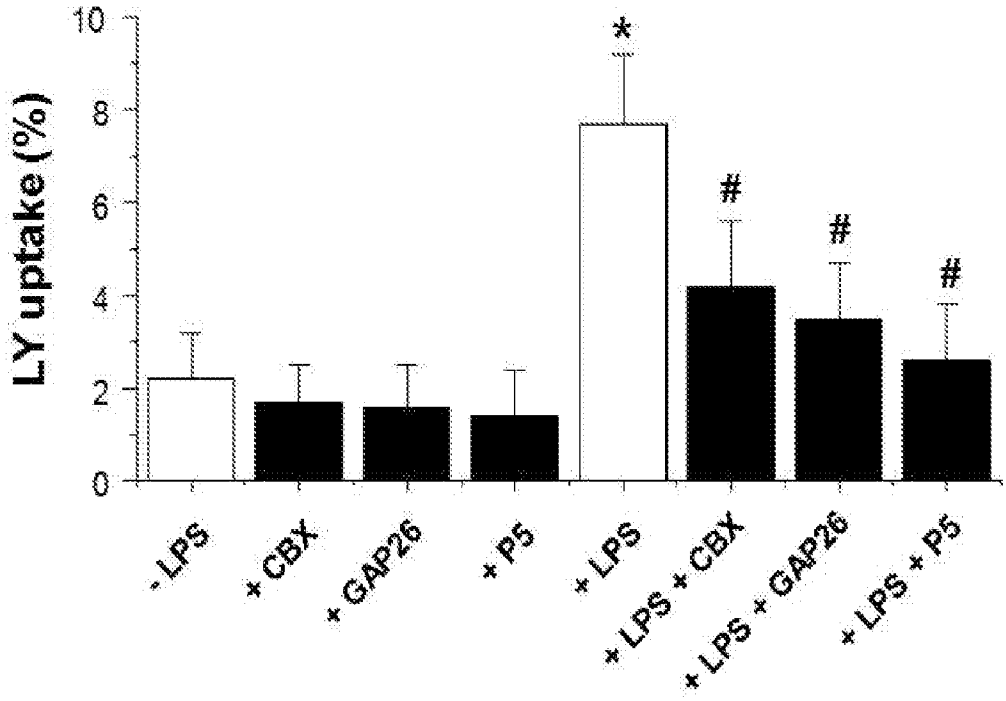

LY uptake (%)
10
8
6
4
2
0
*
#
#
#
- LPS
+ CBX
+ GAP26
+ P5
+ LPS
+ LPS + CBX
+ LPS + GAP26
+ LPS + P5

Fig 8

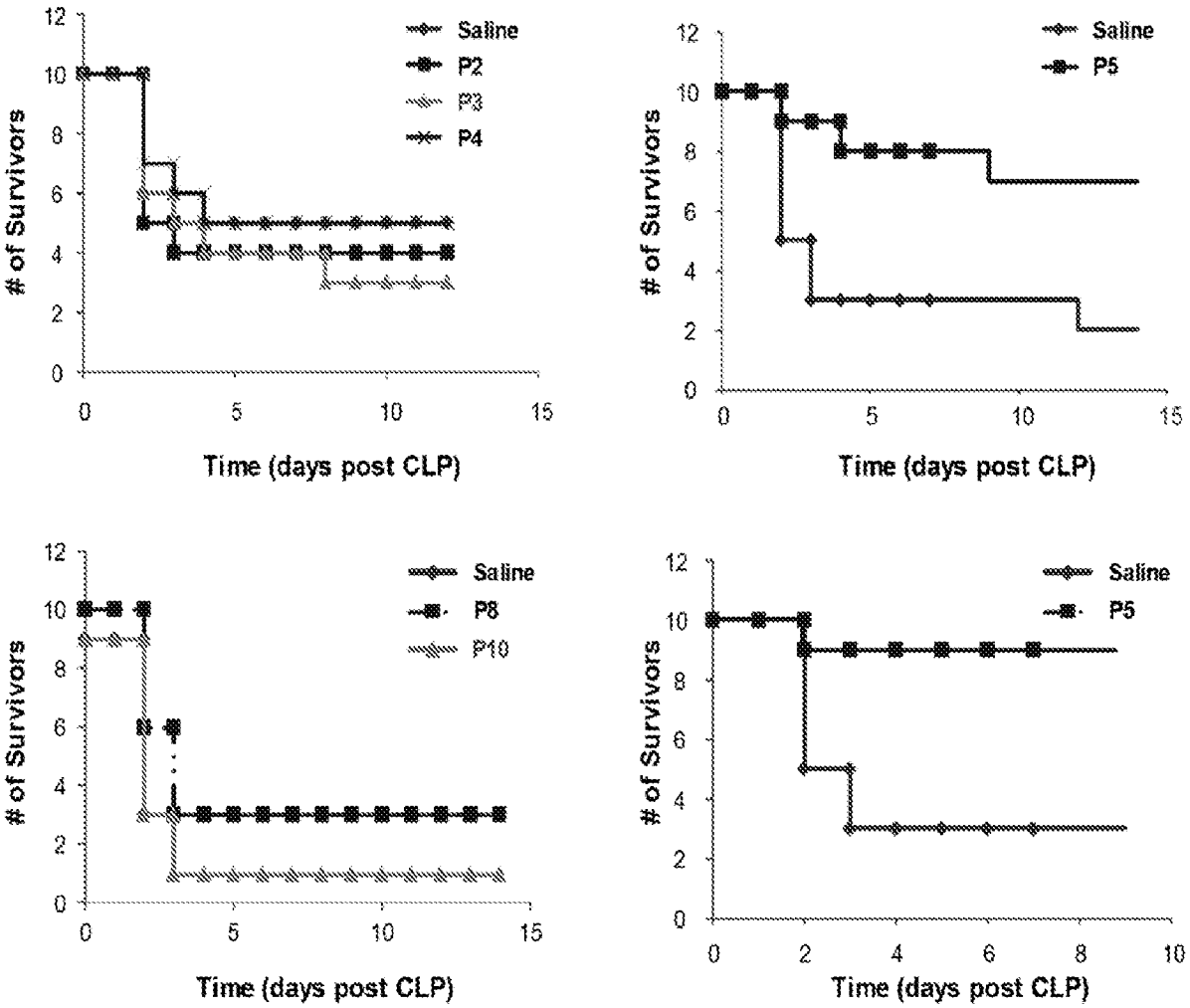

Saline
P2
P3
P4
# of Survivors
Time (days post CLP)
Saline
P5
# of Survivors
Time (days post CLP)
Saline
P8
P10
# of Survivors
Time (days post CLP)
Saline
P5
# of Survivors
Time (days post CLP)

Fig 9

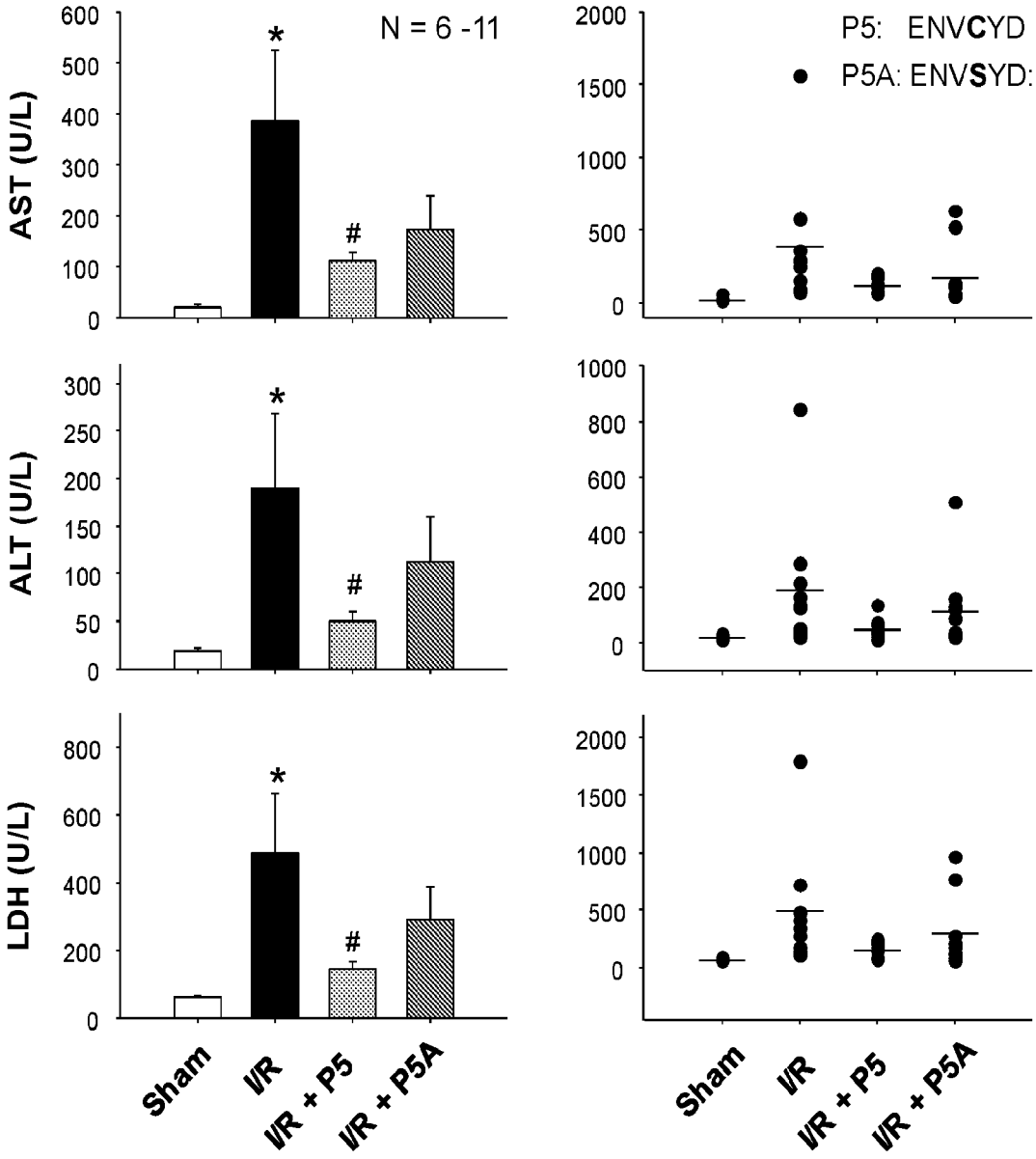

N = 6 -11
P5: ENVCYD
P5A: ENVSYD:
AST (U/L)
ALT (U/L)
LDH (U/L)
Sham
I/R
I/R + P5
I/R + P5A

Fig 10

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/045284

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/08; A61P 29/00; A61P 31/00; A61P 37/02; A61P 7/08 (2016.01)
CPC - A61K 38/177; A61K 38/1793; G01N 2800/26 (2016.11)
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)
IPC - A61K 38/08; A61P 29/00; A61P 31/00; A61P 37/00; A61P 37/02; A61P 7/08
CPC - A61K 38/177; A61K 38/1793; G01N 2800/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/1.4; 514/1.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, PubMed
Search terms used: antagonist, inhibitor, GJA1, AVSD3, CMDR, CX43, EKVP, GJAL, HLHS1, HSS, ODDD, PPKCA, gap junction protein alpha 1, connexin 43, PANX1, MRS1, PX1, UNQ2529, pannexin 1, ischemia reperfusion, sepsis, endotoxemia

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2006/134494 A2 (CODA THERAPEUTICS LIMITED) 21 December 2006 (21.12.2006) entire document | 21-24 |
| P, X | ZHOU et al. "Decreased connexin 43 in astrocytes inhibits the neuroinflammatory reaction in an acute mouse model of neonatal sepsis," Neuroscience Bulletin, 28 September 2015 (28.09.2015), Vol. 31, Iss. 6, Pgs. 763-768. entire document | 1, 2, 3-5, 7-9 |
| A | WOEHRLE et al. "Hypertonic stress regulates T cell function via pannexin-1 hemichannels and P2X receptors," Journal of Leukocyte Biology, 30 September 2010 (30.09.2010), Vol. 88, No 6, Pgs. 1181-1189. entire document | 1-9, 21-26 |
| A | US 2014/0127190 A1 (WISCONSIN ALUMNI RESEARCH FOUNDATION) 08 May 2014 (08.05.2014) entire document | 1-9, 21-26 |
| A | ADAMSON et al. "The role of pannexin1 in the induction and resolution of inflammation," FEBS Letters, 15 March 2014 (15.03.2014), Vol. 588, Pgs. 1416-1422. entire document | 1-9, 21-26 |
| P, A | YANG et al. "Caspase-11 Requires the Pannexin-1 Channel and the Purinergic P2X7 Pore to Mediate Pyroptosis and Endotoxic Shock," Immunity, 10 November 2015 (10.11.2015), Vol. 43, Iss. 5, Pgs. 923-932. entire document | 1-9, 21-26 |

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"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
"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
"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
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 06 November 2016 | 30 NOV 2016 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, VA 22313-1450<br>Facsimile No. 571-273-8300 | Authorized officer<br>Blaine R. Copenheaver<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/045284

**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.: 10-20, 27
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.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/045284

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-19 were searched.