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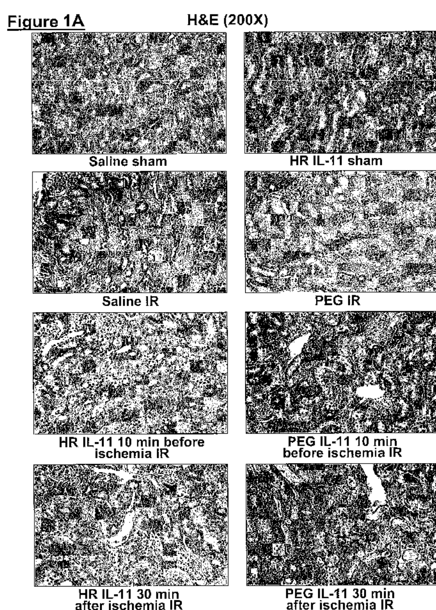
(54) Title: THE USE OF INTERLEUKIN-11 TO PROTECT AGAINST ISCHEMIA AND REPERFUSION INJURY



(57) Abstract: The present invention relates to a method for preventing or treating injury due to ischemia followed by reperfusion by administering IL-11, either in an unmodified or PEGylated form. Administration of the IL-11 would take place prior to the event that caused the injury, in for example, a known surgery, or after the event that caused the injury, in for example, a stroke.

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THE USE OF INTERLEUKIN-11 TO PROTECT AGAINST ISCHEMIA AND REPERFUSION INJURY

CROSS REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S. patent application serial No. 61/667,618 filed July 3, 2012, which is hereby incorporated by reference in its entirety.

FIELD OF INVENTION

This invention is in the field of treating and preventing injury to organs caused by ischemia followed by reperfusion by the administration of interleukin-11.

BACKGROUND OF INVENTION

Ischemia-reperfusion injury ("IR") is caused by the return of blood supply to a tissue (reperfusion) after a period of lack of oxygen (ischemia). During ischemia, there is an absence of oxygen and nutrients normally supplied by the blood. This creates a condition where the return of circulation results in inflammation and oxidative damage, rather than the expected restoration of normal function.

Cell death from ischemia or hypoxia with subsequent reperfusion injury, is a major clinical problem affecting virtually every organ in the body. In fact, post-operative organ dysfunction due to ischemia-reperfusion injury is a severe threat affecting almost all patients undergoing major surgical procedures including cardiac surgery, liver transplantation, liver resection, renal transplantation, lung transplantation, aortic surgery, and major vascular repair. Moreover, injury of one organ due to ischemia or hypoxia with reperfusion is frequently associated with remote organ injury affecting distant organs. For example, patients who develop kidney injury due to renal ischemia and reperfusion frequently develop liver, intestine, and lung dysfunction as well as sepsis leading to extraordinarily high mortality (25-80%). IR injury can also result in patients requiring dialysis.

Renal ischemia-reperfusion injury is especially common, and a frequent cause of acute kidney injury ("AKI") (Ikeda *et al.* (2006)). Ischemic AKI is a clinical problem for patients subjected to major surgical procedures not only involving the kidney, but the liver, heart or aorta as well, and often leads to multi-organ dysfunction and systemic inflammation with extremely high mortality (Jones and Lee (2008)). Currently, the incidence of renal dysfunction after major surgery in high risk patients has been reported to be about 80% (Hoste and Kellum (2007)). Unfortunately, the severity and incidence of AKI has been

increasing without any improvements in therapy or patient survival over the past 50 years (Jo *et al.* (2007)). Thus, there is a need for therapies to treat and prevent injury due to IR. Drugs that can prevent or treat IR injury to the kidney and other organs can potentially reduce mortality rates, organ dysfunction, and the need for dialysis in a variety of clinical settings.

Interleukin -11 ("IL-11") is a 20 kDa multifunctional member of the IL-6-type cytokine family and is a key regulator of megakaryocyte maturation (Goldman *et al.* (2001)). In addition to its hematopoietic properties, recent studies suggest cytoprotective roles for IL-11 (Kuenzler *et al.* (2002)). Specifically, IL-11 administration protects against intestinal, cardiomyocyte and endothelial cell death by producing significant anti-necrotic and anti-apoptotic effects in these cell types (Du and Williams (1997)). IL-11 also attenuates the inflammatory responses in a murine model of LPS-induced sepsis (Trepicchio and Dorner (1998); Trepicchio *et al.* (1997)).

Exogenous administration of native or human recombinant ("HR") IL-11 may be limited by its relatively short half-life of about seven hours due to rapid clearance through urinary excretion, hepatic metabolism and enzymatic degradation (Du and Williams (1997)); Grosfeld *et al.* (1999)). Chemical modification of IL-11 by conjugation to polyethylene glycol ("PEG") reduces IL-11 glomerular filtration and hepatic uptake, therefore prolonging the half-life of IL-11 (Takagi *et al.* (2007)).

SUMMARY OF THE INVENTION

The present invention is based upon the surprising discovery that the administration of interleukin-11 (IL-11) or an IL-11 analog, prior to ischemia/reperfusion injury, mitigates the effects of the injury and protects the organs. Even more surprisingly, IL-11 and IL-11 analog administration after ischemia/reperfusion injury also mitigates the effect of the injury and protects the organs. The latter finding is important in the clinical setting where IR injury cannot always be predicted prior to its occurrence, *e.g.*, before a surgical procedure that may cause an IR injury or before a stroke.

Thus, one embodiment of the present invention is a method of preventing or treating an injury to an organ caused by ischemia, followed by reperfusion, comprising the administration of a therapeutically effective amount of IL-11 or an IL-11 analog, to a subject in need thereof.

Such an injury can be caused by surgery, most usually surgery involving major organs, including but not limited to, the kidney, liver, heart, lung, intestine, and aorta and would include, but is not limited to, coronary bypass, major vascular repair, liver resection,

and transplantation of the kidney, liver, and lung. IR injury can have other causes including, but not limited to, cardiopulmonary bypass during surgery, stroke, liver ischemia, kidney ischemia, aortic occlusion, myocardial occlusion, cardiac arrest, shock and trauma.

In a preferred embodiment, the IL-11 or IL-11 analog is administered prior to the occurrence of the ischemia which causes the injury, which in this embodiment is most likely during surgery. Preferably, the IL-11 protein or IL-11 analog is administered within about one (1) week or less prior to the ischemia or surgery, more preferably within about five (5) days or less prior to the ischemia or surgery, more preferably within about one (1) day or less prior to the ischemia or surgery, more preferably within about six (6) hours or less prior to the ischemia or surgery, more preferably within about sixty (60) minutes or less prior to the ischemia or surgery, more preferably within about thirty (30) minutes or less prior to the ischemia or surgery, and more preferably, within about ten (10) to about fifteen (15) minutes or less prior to ischemia or surgery.

In another preferred embodiment, the IL-11 or IL-11 analog is administered after surgery or after another incident or event has occurred where IR injury would be suspected, including but not limited to, cardiopulmonary bypass during surgery, stroke, liver ischemia, kidney ischemia, aortic occlusion, myocardial occlusion, cardiac arrest, shock and trauma. In this embodiment, the IL-11 protein or IL-11 analog is administered preferably within about one (1) week or less following the surgery or IR injury event, more preferably within about five (5) days or less following surgery or the IR injury incident, more preferably within about one (1) day or less following surgery or the IR injury incident, more preferably within about six (6) hours or less following surgery or the IR injury incident, more preferably within about sixty (60) minutes or less following surgery or the IR injury event, and most preferably within about thirty (30) minutes or less following surgery or the IR injury incident.

In another preferred embodiment, the IL-11 or IL-11 analog is administered following reperfusion of the ischemic organ. In this embodiment, the IL-11 protein or IL-11 analog is administered preferably within about one (1) week or less following reperfusion of the ischemic organ, more preferably within about five (5) days or less following reperfusion of the ischemic organ, more preferably within about one (1) day or less following reperfusion of the ischemic organ, more preferably within about six (6) hours or less following reperfusion of the ischemic organ, more preferably within about sixty (60) minutes or less following reperfusion of the ischemic organ, and most preferably within about thirty (30) minutes or less following reperfusion of the ischemic organ.

In another preferred embodiment, the IL-11 or IL-11 analog is administered at the start of reperfusion of the ischemic organ.

In another preferred embodiment, the IL-11 or IL-11 analog is administered during reperfusion of the ischemic organ.

In another preferred embodiment, the IL-11 or IL-11 analog is administered during surgery to prevent or reduce the severity of acute kidney injury or ischemia-induced injury to other organs or tissues.

The IL-11 for use in the method of the invention can include, but is not limited to, native or wild-type IL-11, recombinant IL-11, and a variant of native or recombinant IL-11, wherein the variant of IL-11 differs in nucleotide sequence or amino acid sequence or both, from native or recombinant IL-11, but retains its ability to protect organs from injury caused IR.

The IL-11 for use in the method of the invention also can include an IL-11 analog that retains its ability to protect organs from injury caused by IR. The IL-11 analog can include, but is not limited to, any form of IL-11 listed herein that is modified by polyethylene glycol, or any other long-acting IL-11 analog. Long-acting IL-11 analogs that can be used in the method of the invention include, but are not limited to, the long-acting, PEGylated IL-11 analogs described herein, depot formulations of the IL-11 proteins, fusion proteins that comprise an IL-11 protein, chemically-modified IL-11 proteins, and polymer-modified IL-11 proteins.

Polymer-modified IL-11 proteins can be prepared by attaching one or more polymers to a variety of amino acids, such as cysteine, lysines, N-terminal amino acids containing one or more amine groups, and non-natural amino acids introduced into the protein or added to the amino terminus or carboxy-terminus of the protein. One example of a polymer-modified IL-11 is described in Takagi *et al.* (2007). The long-acting IL-11 analogs also may contain sugar, starch, fatty acids or lipid groups attached to the IL-11 protein. The polymer, sugar, starch, fatty acid or lipid used for modifying the IL-11 protein can be any polymer, sugar, starch, fatty acid or lipid that confers a half-life that is longer than the half-life of the non-modified IL-11 protein in animals. Examples of methods for incorporating non-natural amino acids into proteins and subsequently modifying the proteins with polymers are described in U.S. Patent No. 7,332,571, U.S. Patent No. 7,385,028, U.S. Patent No. 7,230,068, U.S. Patent No. 8,263,740, U.S. Patent No. 6,586,207, U.S. Patent No. 7,139,665, U.S. Patent No. 7,632,492, and U.S. Patent No. 7,829,659.

The long-acting IL-11 analog also may be an IL-11 fusion protein. One example of a long-acting IL-11 fusion protein is a fusion protein comprising IL-11 fused to an immunoglobulin domain (U.S. Patent No. 7,754,855). Other long-acting fusion proteins known in the art have been prepared by fusing a protein of interest to albumin, transferrin, transferrin receptors, elastin and elastin-like proteins, XTEN sequences, chorionic gonadotrophin carboxy terminal peptides or by adding non-native amino acids to the N-terminus or C-terminus of a protein. Examples of methods to create long-acting fusion proteins are described in U.S. Patent No. 7,553,940, U.S. Patent No. 7,553,941, U.S. Patent No. 8,048,848, U.S. Patent No. 8,252,739, U.S. Patent No. 8,426,166, and in Schellenberger *et al.* (2009) and Cleland *et al.* (2012). The fusion protein used to practice the invention can be any IL-11 fusion protein that confers a half-life that is longer than the half-life of the non-fused IL-11 protein in animals.

Peptides that bind and activate cellular receptors for IL-11 can also be used in the method of the present invention, and can be prepared using methods known in the art, such as by using phage display methods, that have been used to prepare peptides that bind and activate other proteins and cellular receptors. Examples of methods for selecting and using peptides that bind other proteins and cellular receptors are described in U.S. Patent No. 5,773,569, U.S. Patent No. 5,986,047, U.S. Patent No. 6,660,843, U.S. Patent No. 7,166,707, U.S. Patent No. 7,169,905, U.S. Patent No. 7,186,810, U.S. Patent No. 7,189,827, and U.S. Patent No. 7,488,590. Other protein agonists, including antibodies and monoclonal antibodies, that bind and activate the IL-11 receptor (Putoczki and Ernst (2010) are also contemplated by the present invention.

In one preferred embodiment, the IL-11 protein for use in the method of the invention is human IL-11 (Paul *et al.* (1990)). Human IL-11 is synthesized as a 199 amino acid precursor protein containing an N-terminal 21 amino acid signal sequence, which is cleaved from the mature 178 amino acid protein. The mature IL-11 protein begins with proline at amino acid position 22. In another preferred embodiment, the IL-11 protein for use in the method of the invention is a recombinant human desPro IL-11 (IL-11 missing the N-terminal proline residue present in the native protein). In another preferred embodiment, the IL-11 protein for use in the method of the invention is a human IL-11 protein that is modified with a chemical moiety including, but not limited to, a polymer, a sugar, a starch, a fatty acid or a lipid. In another preferred embodiment, the IL-11 protein for use in the method of the invention is human IL-11 and the protein is modified with polyethylene glycol or PEG. In another embodiment, the IL-11 protein for use in the method of the invention comprises a

cysteine variant of IL-11, such as any of the IL-11 cysteine variants described in PCT/US98/14497, U.S. Patent No. 7,253,267, U.S. Patent No. 7,495,087, and U.S. Patent No. 8,133,480. These patents describe IL-11 cysteine variants compromising one or more added cysteine residues, wherein the added cysteine residue(s) has been substituted for one of more amino acids in the IL-11 protein sequence, or wherein the added cysteine residue(s) has been added preceding the first amino acid of the mature IL-11 protein or following the last amino acid of the IL-11 protein. The added cysteine residue(s) in the IL-11 cysteine variants may be modified with one or more polymers such as PEG. In another preferred embodiment, the IL-11 protein for use in the method of the invention is a human IL-11 variant wherein a cysteine residue is added following the last amino acid of the IL-11 protein. In another preferred embodiment the IL-11 protein for use in the method of the invention comprises an IL-11 variant wherein a cysteine residue is added following the last amino acid of the protein and wherein the protein is further modified with one or more PEGs. Preferably, the IL-11 cysteine variant is modified with a 40 kDa-PEG at the added cysteine residue. The PEG used to modify the IL-11 protein can be any PEG known in the art, including, but not limited to, a linear PEG and a branched PEG. PEGs are available in different sizes ranging from about 2 kDa to about 80 kDa, and all can be used to create IL-11 analogs for use in practicing the method of the invention.

Therapeutically effective doses of the IL-11 proteins for use in the method of the invention range from about 1 nanogram of IL-11 per kilogram of body mass of the subject, to about 10 milligrams of IL-11 per kilogram of body mass, more preferably from about 1 microgram of IL-11 per kilogram of body mass, to about 1 milligram of IL-11 per kilogram of body mass, and even more preferably from about 10 micrograms of IL-11 per kilogram body mass to about 100 micrograms of IL-11 per kilogram of body mass.

All major organs can be protected from IR injury by the administration of IL-11 and would include, but are not limited to, the kidneys, liver, lungs, intestines, heart, and blood vessels.

In a preferred embodiment, the subject is a mammal and in the most preferred embodiment, the mammal is a human.

BRIEF DESCRIPTION OF THE FIGURE

For the purpose of illustrating the invention, there are depicted in drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1 shows representative photomicrographs for hematoxylin and eosin staining (Figure 1A, magnification 200X), TUNEL staining (Figure 1B, representing apoptotic nuclei, magnification 200X) and immunohistochemistry for neutrophil infiltration (Figure 1D, magnification 400X) of kidney sections of mice. In Figure 1D, the black arrows denote neutrophils. Photographs are representative of 3-5 independent experiments. Figure 1C is a graph showing the number of apoptotic cells per 200X field in the kidneys of mice after renal IR (30 minutes of ischemia and 24 hours of reperfusion). Figure 1E is a graph showing the number of infiltrated neutrophils per 400X field in the kidneys of mice after renal IR (30 minutes of ischemia and 24 hours of reperfusion). *$P<0.05$ vs. vehicle-treated mice subjected to renal IR. Error bars represent 1 SEM.

Figure 2 is a Western blot of representative poly(adenosine diphosphate-ribose) polymerase (PARP) and caspase-3 fragmentation (N=3-4 for each group) as indices of HK-2 cell apoptosis. β-actin served as internal loading controls.

Figure 3 is representative images for sphingosine kinase (SK1 and SK2) mRNA (RT-PCR) and protein (immunoblotting) expression in HK-2 cells treated with saline (vehicle for HR IL-11) or with 100 ng/mL HR IL-11 (N=4 for each group).

Figure 4 are graphs showing the lactate dehydrogenase (LDH) released after H_2O_2-induced necrosis in IEC-6 cells (expressed as a percentage of total LDH released). Figure 4A show the results of cells pretreated with WT HR IL-11 and then treated with 1mM H_2O_2. Figure 4B shows the results of cells pretreated with WT HR IL-11 or PEGylated IL-11 and then treated with 500 μM H_2O_2.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the methods of the invention and how to use them. Moreover, it will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of the other synonyms. The use of examples

anywhere in the specification, including examples of any terms discussed herein, is illustrative only, and in no way limits the scope and meaning of the invention or any exemplified term. Likewise, the invention is not limited to its preferred embodiments.

The term "interleukin-11" and "IL-11" will be used interchangeably and would include native, or wild-type IL-11 isolated directly from mammalian cells and tissues by any method known in the art, such as affinity column purification, IL-11 synthesized via chemical means or by recombinant DNA technology, by any method known in the art, or IL-11 made by any as yet unknown method. It should be noted that when the designation "WT HR IL-11" or "WT IL11" is used in some examples and figures this denotes human recombinant IL-11 that has no variations in the amino acid sequence from native IL-11, except for the absence of the N-terminal proline residue present in native human IL-11. IL-11 lacking the N-terminal proline residue is referred to as desPro IL-11.

The term "IL-11 variant" or "variant" is a protein with variations in either DNA sequence or amino acid sequence or both, from native IL-11, that does not affect the ability of the IL-11 to protect organs from injury due to ischemia-reperfusion. One such variation is an IL-11 protein with a cysteine residue added following the last amino acid of the native or recombinant protein. Another such amino acid variant is desPro IL-11.

The term "IL-11 analog" or "analog" refers to an IL-11 protein that contains any other chemical modification that causes the protein to be chemically or structurally different from native IL-11, but does not affect the ability of the IL-11 to protect organs from injury due to ischemia-reperfusion. Examples of such chemical modifications include, but are not limited to, modifications of the IL-11 protein with one or more polymers, sugar, starches, fatty acids, or lipids, modifications of the IL-11 protein with any chemical compound, and modification of the IL-11 protein by addition or deletion of amino acids.

The terms "PEGylated interleukin-11", "PEGylated IL-11", and "PEG IL-11" will be used interchangeably and will include any and all IL-11 proteins, IL-11 variants, and IL-11 analogs defined above that are chemically modified by conjugation of polyethylene glycol ("PEG") to an amino acid residue.

The term "injury" would refer to tissue or organ damage, and includes any alteration in tissue or organ structure, cell viability or function. The terms "injury from ischemia followed by reperfusion", "injury from IR", "IR injury" and the like would refer to the tissue damage caused when blood supply returns to a tissue after a period of lack of oxygen.

The terms "prevent", "prevention", and the like refer to acting prior to the tissue and/or organ damage to minimize the damage, to prevent the damage from developing or to minimize the extent of the damage or to slow its course of development.

The terms "treat", "treatment", and the like refer to a means to slow down, relieve, ameliorate, or alleviate the damage or injury to the tissues and/or organs or reverse the damage after its onset.

The term "protect", "protection" and the like refer to a means to ameliorate the damage from the injury or stop the injury to the organ and/or tissue from occurring.

The term "subject" as used in this application means an animal with an immune system such as avians and mammals. Mammals include canines, felines, rodents, bovine, equines, porcines, ovines, and primates. Avians include, but are not limited to, fowls, songbirds, and raptors. Thus, the invention can be used in veterinary medicine, *e.g.*, to treat companion animals, farm animals, laboratory animals in zoological parks, and animals in the wild. The invention is particularly desirable for human medical applications

The term "in need thereof" would be a subject known or suspected of having an injury caused by ischemia and reperfusion. A subject in need thereof may have had or will be having surgery, including but not limited to, surgery involving the heart, lungs, liver, kidneys, intestines, or aorta. Alternatively, the subject in need thereof may have suffered a stroke, liver ischemia, kidney ischemia, aortic occlusion, myocardial occlusion, cardiac arrest, shock or trauma, or any other medical condition that results in ischemia to one or more tissues or organs.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to cause an improvement in a clinically significant condition in the subject, or delays or minimizes or mitigates one or more symptoms associated with the injury, or results in a desired beneficial change of physiology in the subject.

Interleukin-11 (IL-11) and PEGylated IL-11

Interleukin-11 or IL-11, a member of the IL-6-type cytokine family, was first identified from bone marrow-derived stromal cells. It is a key regulator of hematopoiesis and promotes megakaryocyte maturation (Reynolds (2000)). IL-11, as well as its receptors, is expressed in many tissues and cell types (Du and Williams (1997)). In addition to its hematopoietic properties, recent studies suggest a cytoprotective role for IL-11 (Du and Williams (1997)). In several organs including the heart, intestine and endothelial cells, IL-11

administration has been shown to attenuate necrotic as well as apoptotic cell death (Du and Williams (1997)). In addition to its anti-apoptotic and anti-necrotic properties, IL-11 administration reduces inflammatory responses in lipopolysaccharide-treated mice (Sheridan *et al* (1999); Trepicchio *et al.* (1996)), macrophage inflammation (Trepicchio *et al.* (1997)), nephrotoxic nephritis (Lai *et al.* (2001)), and T-cell mediated liver injury (Bozza *et al.* (1999)). IL-11 treatment following coronary occlusion partially reduces cardiac fibrosis, cardiac cell death and cardiac remodeling (Kimura *et al* (2007); Obana *et al* (2010); Fujio *et al* (2011))

A clinical formulation of recombinant human IL-11 (Oprelvekin) is already approved by the FDA to treat chemotherapy-related thrombocytopenia. Oprelvekin is produced in *Escherichia coli (E. coli)* by recombinant DNA technology. The protein has a molecular mass of approximately 19,000 daltons, and is non-glycosylated. The polypeptide is 177 amino acids in length and differs from the 178 amino acid length of native IL-11 only in the lack of the amino-terminal proline residue (referred to as desPro IL-11). This alteration has not resulted in measurable differences in bioactivity either *in vitro* or *in vivo.*

Both native or wild-type, and recombinant IL-11 can be used in the current invention to protect kidneys and other organs from IR injury. The recombinant IL-11 can be human.

Covalent modification of proteins with polyethylene glycol or PEG have proven to be a very useful method to extend the circulating half-lives of proteins and several PEGylated proteins are now approved for use in humans (Cox *et al.* (2007)). Long-acting IL-11 analogs would not require frequent dosing and could provide significant treatment advantages in a clinical setting. In addition to improving protein half-life, PEG modification can increase protein solubility and stability and decrease protein immunogenicity (Takagi *et al.* (2007)).

Although PEGylation may increase the half-life of IL-11, it may also result in substantial decrease in biological activity or potency due to its steric hindrance (Takagi *et al.* (2007)).

The most commonly employed method for PEGylating proteins uses compounds that attach PEG to free amines, typically at lysine residues or at the N-terminal amino acid. A critical limitation of this approach is that proteins typically contain several lysines, in addition to the N-terminal amino acid. The PEG moiety can attach to the protein at any of the available free amines, resulting in a heterogeneous product mixture consisting of mono-, di-, tri-, etc, PEGylated species modified at different lysine residues and the N-terminus. The different PEGylated species often possess different intrinsic biological activities. This can present problems when developing a PEGylated protein therapeutic because predictability of

biological activity and manufacturing reproducibility are crucial for regulatory approval. Many amine-PEGylated proteins are unsuitable for commercial use because of low specific activities. Biological activities of amine-PEGylated proteins often are reduced 10-100-fold relative to the non-modified protein. Inactivation results from covalent modification of one or more amino acids required for biological activity or from covalent attachment of the PEG moiety near the active site or ligand binding site of the protein.

By using site specific PEGylation and conjugating PEG to a unique, engineered cysteine residue in IL-11, these problems of product heterogeneity and loss of biological activity typical of amine-PEGylation were overcome. Site specific PEGylation allows a protein to be selectively modified with PEG at a unique predetermined site. By targeting the PEG molecule to an optimal site in a protein, it is possible to create PEGylated proteins that are homogenously modified and have no significantly loss of biological activity. A novel, long acting IL-11 analog that has undergone cysteine residue specific chemical modification of the protein with PEG was produced (Example 1A) that has increased potency and half- life of IL-11 (by more than 10 fold to approximately 300-500 minutes in rats). This novel, long-acting IL-11 analog is described and claimed in at least U.S. Patent Nos. 7,253,267, 7,495,087, and 8,133,480, all issued to George Cox, III, and all incorporated herein by reference in their entirety.

IL-11 Protects Against Ischemia-Reperfusion Injury

Acute kidney injury (AKI) is a frequent and disastrous clinical complication with high mortality, morbidity and cost (Faubel (2009); Jones and Lee (2008)). Renal IR injury is a major cause of AKI for patients subjected to surgical procedures involving the kidney, heart, liver or aorta. Although not completely understood, renal tubular necrosis, apoptosis and inflammation during and after renal IR contribute significantly to the pathogenesis of ischemic AKI (Bonventre and Weinberg (2003)).

The basis of this invention is the unexpected discovery that recombinant human IL-11, as well as a novel PEGylated IL-11, attenuates renal tubular cell death *in vivo,* as well as *in vitro,* by reducing necrosis, apoptosis and inflammation, and that IL-11 has protective effects against renal IR injury in mice and humans. In IR injured mouse kidneys, IL-11 attenuated renal tubular necrosis (Jablonski renal injury score) as well as apoptosis (TUNEL staining) (Example 3; Figures 1A and 1C). Additionally, IL-11 treatment reduced the influx of pro-inflammatory neutrophils after renal IR in mouse kidneys (Example 3: Figure 1D). In

human kidney cells, IL-11 also reduced necrosis (LDH release) as well as apoptosis (PARP/caspase 3 fragmentation) (Example 4; Figure 2). Therefore, it was concluded that exogenous administration of IL-11 provides powerful renal protection against ischemic AKI by targeting all three pathways of cell death: necrosis, apoptosis and inflammation.

Perhaps a more unexpected and more important discovery is that there was a significant protective effect by HR IL-11 or PEGylated IL-11 therapy not only when given before renal ischemia, but also when administered after renal ischemia (Examples 2 and 3). The latter observation makes the use of IL-11 therapy particularly effective for a diverse group of patients at risk for ischemic AKI because, while ischemia can be predicted in many complicated surgical procedures leading to renal injury, a significant number of patients present symptoms after renal ischemic injury has already occurred. Post-ischemic therapy for AKI is a significant clinical advance as not all ischemic AKI can be anticipated prior to surgical procedures or even arise from surgical procedures, but can arise from unexpected events, such as a stroke, aortic occlusion, myocardial occlusion, cardiac arrest, shock or trauma.

After IL-11 binds to the IL-11 receptor, the ligand-receptor complex interacts with a common receptor subunit, glycoprotein 130 (gp130), leading to gp130-associated kinase-mediated tyrosine phosphorylation (Fujio *et al.* (2011)). The cytoprotective mechanisms of IL-11 leading to reductions in necrosis, inflammation as well as apoptosis have been investigated in other cell types. In cardiac myocytes, IL-11 reduces injury and fibrosis by Janus Kinase - Signal Transducer and Activator of Transducer 3 (JAK-STAT3) pathway activation (Fujio *et al.* (2011); Kimura *et al.* (2007); Obana *et al.* (2010)). In vascular endothelial and intestinal epithelial cells, IL-11 protects against oxidant induced necrosis and apoptosis via mechanisms involving ERK MAPK, Akt and/or induction of HSP25 (Naugler *et al.* (2008); Ropeleski *et al.* (2003); Waxman *et al.* (2003)).

While not being bound by any theory, the data set forth herein suggest that IL-11 produces renal protection by direct induction of sphingosine kinase-1 (SK1) via nuclear translocation of hypoxia inducible factor 1α (HIF-1α). IL-11-mediated induction of SK1 has not been described previously and represents a novel development in the understanding of cytoprotective mechanisms of IL-11 administration.

SK is a multifunctional lipid kinase that phosphorylates sphingosine to form sphingosine 1-phosphate (S1P). Of the two forms of SK, SK1 is a cytosolic enzyme that migrates to the plasma membrane or to the nucleus upon activation (Hait *et al.* (2006);

Leclercq *et al.* (2006)). SK1 is a well-known mediator of tissue protection (including protection against IR injury), growth and survival (Liu *et al.* (2002)). Overexpression of SK1 is protective in acute lung injury (Wadgaonkar *et al.* (2009)). Furthermore, in cardiac IR injury, SK1 activation protects against cardiomyocyte death and SK1-deficient cardiomyocytes had increased injury after ischemia (Vessey et al. (2005)). It has also been previously demonstrated a renal protective role of SK1 as well as $S1P_1$ receptor activation (Bonventre and Weinberg (2003); Jo *et al.* (2009); Kim *et al.* (2007); Kim *et al.* (2010)). Overall, activation of SK1 produces anti-necrotic, anti-inflammatory and anti-apoptotic effects in several organs and cell types.

It has also been shown herein that IL-11 treatment not only induces SK1 (Example 5) but that the renal protective effects of IL-11 also are dependent on SK1, as mice deficient in SK1 enzyme were not protected against renal IR injury with either HR IL-11 or PEGylated IL-11 treatment (Example 6). Again while not being bound by any theories, it appears that IL-11-mediated SK1 induction enhances the synthesis of endogenous S1P in the kidney. S1P is a potent lipid signaling molecule that can activate 5 S1P receptors (S1PR) to regulate cell growth, cell survival and modulation of inflammation (Allende *et al.* (2004)(1); Chae *et al.* (2004); Venkataraman *et al.* (2006)). $S1P_1R$ activation in particular has been shown to produce tissue protection by attenuating T-lymphocyte-mediated inflammation. Activation of the $S1P_1Rs$ on endothelial cells also reduces vascular permeability, hence better preserving the integrity of the vascular endothelial cell barrier function (Allende *et al.* (2003)). Furthermore, direct renal tubular protective effects of $S1P_1R$ activation are mediated by activation of the Akt and ERK pathways (Bajwa *et al.* (2010)). Therefore, both SK1→$S1P_1R$ as well as IL-11→IL11R→gp130 pathways can activate cytoprotective ERK and Akt signal transduction.

The data herein also implicate an important role for HIF-1α in mediating the induction of SK1 after IL-11 treatment. HIF-1 is a heterodimeric transcription factor composed of an α and a β subunit (Rosenberger *et al.* (2008); Schodel *et al.* (2009)). Under normoxic conditions, prolyl-hydroxylation and ubiquitination of the oxygen-dependent degradation domain of HIF-1α results in rapid HIF-1α degradation. With hypoxia or ischemia, HIF-1α stabilizes and interacts with HIF-1β forming the HIF-1 heterodimer. Nuclear HIF-1 translocation allows binding to the hypoxia-responsive element with subsequent induction of several cytoprotective genes. Consistent with this proposed

pathway, previous studies have demonstrated that HIF-1α activation protects against renal IR injury (Schodel *et al.* (2009)).

The data set forth herein show that HR IL-11 or PEGylated IL-11 caused increased HIF-1α nuclear translocation, SK1 induction and enhanced SK1 activity in HK-2 cells (Examples 5 and 7; Figure 3). Thus, without being bound by any theory, it is proposed that IL-11 receptor activation causes binding of hypoxia inducible factor-1α to hypoxia response elements of the SK1 promoter, leading to increase in SK1 protein synthesis and activity. Consistent with this hypothesis, the data also show that selective HIF-1α blockers (2-ME or YC-1) prevented IL-11 -mediated induction of SK1 in HK-2 cells (Example 7). 2-ME is a natural metabolite of estrogen that is known to inhibit HIF-1α at the level of translation (Mooberry (2003)). YC-1 is a selective inhibitor of HIF-1α transcriptional activity ((Yeo *et al.* (2003)).

In summary, the findings that IL-11-mediated SK1 induction leads to reduced renal injury represent a novel use for IL-11 therapy.

Additionally, these findings may lead to new therapeutic approaches with a drug that can reduce all three pathways of renal cell death (necrosis, apoptosis and inflammation) to lessen the clinical perils from AKI and have implications in organ protection strategies beyond the kidney.

Indeed, as shown by the data herein, IL-11 also has a protective effect in the liver which has been injured by ischemia/ reperfusion (Example 8) and decreases necrosis in intestinal epithelial cells (Example 9).

Subjects Benefitting from IL-11 Administration

It has been surprisingly discovered the administration of IL-11, whether native, recombinant or a variant, whether or not an IL-11 analog, or whether or not modified by PEG, protects organs from IR injury. IR injury most often is a result of surgery involving major organs, such as the kidney, liver, lungs, heart, aorta or intestines. Thus, any subject who is known to be undergoing surgery involving these organs would benefit from administration of IL-11 prior to the surgical procedure.

Even more surprising and more clinically significant is the discovery that the administration of IL-11 after the cause of the IR injury, *e.g.*, surgery, stroke, cardiac arrest, trauma, and shock, will also protect the organs from damage caused by IR. Additionally, since it is not always readily apparent that an IR injury has occurred, a subject who has

suffered any type of event that would be suspected of causing ischemia, such as trauma, stroke, or cardiac arrest, would benefit from the administration of IL-11, as well as those patients subject to a surgical procedure in which the IL-11 was not administered prior to the surgical procedure.

Pharmaceutical Compositions and Methods of Administration

The present invention encompasses the administration of IL-11 and IL-11 variants, and IL-11 analogs, including PEGylated IL-11 variants and analogs. Preferred methods of administration include oral; mucosal, such as nasal, sublingual, vaginal, buccal, or rectal; parenteral, such as subcutaneous, intravenous, intraperitoneal, intradermal, bolus injection, intramuscular, or intraarterial; or continuous infusion; or transdermal administration to a subject. Thus, the IL-11must be in the appropriate form for administration of choice.

Such compositions for administration may comprise a therapeutically effective amount of the IL-11, IL-11 variants or IL-11 analogs, and a pharmaceutically acceptable carrier. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human, and approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. "Carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, bulking agents such as trehalose, mannitol, sorbitol, and sucrose, amino acids such as glycine, histidine, methionine, and arginine, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. Detergents commonly used in pharmaceutical compositions include polysorbate-20, polysorbate-80, pluronic acid F68, sodium dodecyl sulfate, and sodium lauryl sulfate.

These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations, cachets, troches, lozenges, dispersions, suppositories, ointments, cataplasms (poultices), pastes, powders, dressings, creams, plasters, patches, aerosols, gels, liquid dosage forms suitable for parenteral administration to a patient, and sterile solids (*e.g.*, crystalline or amorphous solids) that can be reconstituted to provide liquid dosage forms suitable for parenteral administration to a patient. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable form of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

Pharmaceutical compositions adapted for oral administration may be capsules, tablets, powders, granules, solutions, syrups, suspensions (in non-aqueous or aqueous liquids), or emulsions. Tablets or hard gelatin capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, stearic acid or salts thereof. Soft gelatin capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols. Solutions and syrups may comprise water, polyols, and sugars. An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract. Thus, the sustained release may be achieved over many hours and if necessary, the active agent can be protected from degradation within the stomach. Pharmaceutical compositions for oral administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient over a prolonged period of time.

Pharmaceutical compositions adapted for nasal and pulmonary administration may comprise solid carriers, such as powders, which can be administered by rapid inhalation through the nose. Compositions for nasal administration may comprise liquid carriers, such as sprays or drops. Alternatively, inhalation directly through into the lungs may be accomplished by inhalation deeply or installation through a mouthpiece. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient.

Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain anti-oxidants, buffers, bacteriostats, and solutes that render the compositions substantially isotonic with the blood of the subject. Other components which may be present in such compositions include water, alcohols, polyols, glycerine, and vegetable oils. Compositions adapted for parental administration may be presented in unit-dose or multi-dose containers, such as sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile carrier, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets. Suitable vehicles that can be used to provide parenteral dosage forms of the invention are well known to those skilled in the art. Examples include: Water for Injection USP; aqueous vehicles such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, and Lactated Ringer's Injection; water-miscible vehicles such as ethyl alcohol, polyethylene glycol, and polypropylene glycol; and non-aqueous vehicles such as corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

Selection of a therapeutically effective dose will be determined by the skilled artisan considering several factors which will be known to one of ordinary skill in the art. Such factors include the particular form of the IL-11 protein, and its pharmacokinetic parameters such as bioavailability, metabolism, and half-life, which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether the administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus, the precise dose should be decided according to the judgment of the person of skill in the art, and each patient's circumstances, and according to standard clinical techniques.

Examples

The present invention may be better understood by reference to the following non-limiting examples, which are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed to limit the broad scope of the invention.

Example 1- General Materials and Methods

A. Interleukin-11 preparation

Human recombinant IL-11 (HR IL-11) and cysteine-residue specific PEG-conjugated (PEGylated IL-11) were synthesized at Bolder BioTechnology, Inc., Boulder, CO. IL-11 and IL-11 (*179C) proteins were expressed as fusion proteins in *E. coli* strain ER2566 using the pTYB11 expression plasmid (New England Biolabs, Beverly, MA) essentially as described in U.S. Patent Nos. 7,495,087 and 8,133,480. IL-11 (*179C) is an IL-11 analog containing a cysteine residue added following the last amino acid of the native protein. The expressed fusion protein comprises an N-terminal chitin binding domain joined to a yeast intein sequence followed by desProIL-11 or desProIL-11 (*179C). *E. coli* expression of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to the cultures. Following induction, the induced cells were lysed and the fusion protein captured on a chitin affinity column (New England Biolabs). The chitin column was washed with buffer containing 50 mM dithiothreitol to activate the intein domain, which cleaves IL-11 from the fusion protein. The cleaved IL-11 proteins were eluted from the column and purified by S-Sepharose column chromatography. The purified IL-11 (*179C) protein was modified with a branched 40 kDa maleimide-PEG obtained from Nippon Oil and Fat Corporation (Irvine, CA) and the PEGylated protein purified from unreacted protein and unreacted PEG by S-Sepharose column chromatography. By RP-HPLC and SDS-PAGE analyses, both proteins were approximately 95% pure.

B. Murine model of Renal IR injury

After Institutional Animal Care and Use Committee (Columbia University) approval, adult male C57BL/6 (Harlan, Indianapolis, IN) as well as $SK1^{-/-}$ or $SK2^{-/-}$ mice (on a C57BL/6 background, kindly provided by Dr. R. L. Proia, NIH, Bethesda, MD, (as described Allende *et al.* (2004) and Mizugishi *et al.* (2005)) were subjected to 30 minutes of renal IR followed by 24 hours of reperfusion as described in Kim *et al.* (2010)(2) and Kim *et al.* (2009).

C. Cell culture

Human kidney proximal tubule cells (HK-2 cells) (ATCC, Manassas, VA) were used for assessments at the cellular level.

Rat intestinal epithelial cells (IEC-2 cells) (ATCC, Manassas, VA) were also used for assessments at the cellular level.

Cells were cultured as directed by the source.

D. Statistical analysis

The data were analyzed with Student's *t*-test when comparing means between two groups or one-way ANOVA plus Tukey's *post hoc* multiple comparison test when comparing multiple groups. Two-way ANOVA plus Bonferroni posttest was used to test the effects of sham operation or renal IR injury on different mouse strains or treatment groups. The ordinal values of the renal injury scores were analyzed by the Mann–Whitney nonparametric test. In all cases, a probability statistic < 0.05 was taken to indicate significance. All data are expressed throughout the text as means ± SEM.

Example 2- The Renal Protective Effect of IL-11 Administration

In order to test whether human recombinant (HR) IL-11 or PEGylated IL-11 administration before and after renal IR injury protects against injury, mice with renal IR were administered with the IL-11 prior to, and after renal injury, and tests for renal function performed.

Materials and Methods

Mice as described in Example 1B were pretreated with saline (vehicle for HR IL-11), PEG (vehicle for PEGylated IL-11), HR IL-11 (0.1-1 mg/kg, i.p.) or long acting PEGylated IL-11 (0.1-1 mg/kg, i.p.) (both as described in Example 1A), 10 minutes prior to renal ischemia or sham-operation.

Separate cohorts of mice as described in Example 1B were treated with saline, PEG, HR IL-11 (1 mg/kg, i.p.) or PEGylated IL-11 (1 mg/kg, i.p) (both as described in Example 1A) 30 minutes or 60 minutes after reperfusion of the ischemic kidney.

Plasma samples were collected 24 hours after IR injury to examine the severity of renal dysfunction by measurements of plasma creatinine, measured with an enzymatic creatinine reagent kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA) (Slot (1965)). Unlike the Jaffe method, this method of creatinine measurement largely eliminates the interferences from mouse plasma chromagens.

Elevated plasma creatinine values are indicative of renal dysfunction. The kidney is the primary organ for filtering creatinine out of the blood and into the urine (via kidney

glomerular filtration and proximal tubular secretion). Levels of creatinine in blood and urine can be used to calculate creatinine clearance. Plasma creatinine levels are directly related to the kidney glomerular filtration rate and are a commonly used indicator of kidney function. An increase in plasma creatinine levels indicates that kidney glomerular filtration is impaired, *i.e.*, lower than normal glomerular filtration rate, and that kidney damage has occurred. When the glomerular filtration rate becomes too low, patients require dialysis.

Results

Plasma creatinine values were similar between all sham-operated (anesthesia, laparotomy, right nephrectomy and recovery) mice 24 hours after surgery, whether saline-treated (Cr=0.52±0.03 mg/dL, N=4), PEG-treated (Cr=0.51±0.03 mg/dL, N=3), HR IL-11-treated (Cr=0.45±0.03 mg/dL, N=3), or PEGylated IL-11-treated (Cr=0.46±0.03 mg/dL, N=3).

Plasma creatinine significantly increased in saline- or PEG-treated mice subjected to 30 minute renal IR compared to sham-operated mice. Pretreatment with HR IL-11 or PEGylated IL-11 (10 minutes before renal ischemia, 0.1-1mg/kg, N=5-6 per group) partially but significantly attenuated the increases in plasma creatinine in mice after renal IR (Table 1).

Table 1 - Renal protection with pre-ischemic IL-11 treatment

Plasma creatinine levels from wild mice subjected to sham-surgery or to renal ischemia and reperfusion (IR). Data are means ± SEM. [*]$P<0.05$ vs. vehicle-treated mice subjected to sham-surgery. [#]$P<0.05$ vs. vehicle-treated mice subjected to renal IR.

Treatment	Creatinine (mg/dL)
Saline sham	0.523 ±0.03
HR IL-11 (1 mg/kg) sham	0.453 ±0.03
Saline IR	2.534 ±0.13*
HR IL-11 (0.1mg/kg) IR	1.851 ±0.04*, #
HR IL-11 (0.3mg/kg) IR	1.495 ±0.07*, #
HR IL-11 (1.0 mg/kg) IR	1.362 ±0.04*, #
PEG sham	0.515 ±0.03
PEG IL-11 (1 mg/kg) sham	0.462 ±0.04
PEG IR	2.386 ±0.04*
PEG IL-11 (0.1mg/kg) IR	1.551 ±0.07*, #
PEG IL-11 (0.3mg/kg) IR	1.155 ±0.17*, #

PEG IL-11 (1.0 mg/kg) IR	1.121 ±0.08*, #

IL-11 treatment after renal reperfusion (after completion of renal ischemia) also protected against renal IR injury. Table 2 shows that HR IL-11 or PEGylated IL-11 (1 mg/kg, N=5-6 per group) given 30 or 60 minute after reperfusion significantly attenuated the increase in plasma creatinine and was protective against renal IR injury. Sham-operated mice were treated either with vehicle (saline or PEG) or with 1 mg/kg HR IL-11 or PEGylated IL-11 (N=4 per group).

Table 2- Renal protection with post-ischemic IL-11 treatment

Plasma creatinine levels from wild mice subjected to sham-surgery or to renal ischemia and reperfusion (IR). Data are means ± SEM. *P<0.05 vs. vehicle-treated mice subjected to sham-surgery. #P<0.05 vs. vehicle-treated mice subjected to renal IR.

Treatment	**Creatinine (mg/dL)**
Saline sham	0.472 ±0.05
HR IL-11 (1 mg/kg) sham	0.48 ±0.04
Saline IR	2.534 ±0.13*
HR IL-11 (1mg/kg) injected 30 min after renal reperfusion	1.625 ±0.07*, #
HR IL-11 (1mg/kg) injected 60 min after renal reperfusion	1.857 ±0.07*, #
PEG sham	0.472 ±0.05
PEG IL-11 (1 mg/kg) sham	0.48 ±0.04
PEG IR	2.386 ±0.04*
PEG IL-11 (1mg/kg) injected 30 min after renal reperfusion	1.438 ±0.07*, #
PEG IL-11 (1mg/kg) injected 60 min after renal reperfusion	1.663 ±0.04*, #

Pre-or post-ischemic treatment with either HR IL-11 or PEGylated IL-11 significantly attenuated the increases in plasma creatinine after renal IR, indicating that the proteins protect the kidney from IR injury. Lower plasma creatinine levels in the IL-11 and PEGylated IL-11 treated mice indicate that their kidney glomerular filtration rates are higher than those of vehicle-treated mice, and closer to the glomerular filtration rates in normal (untreated) mice.

Example 3- Further Evidence of the Renal Protective Effect of IL-11 Administration

In order to further test whether HR IL-11 or PEGylated IL-11 administration before and after renal IR injury protects against injury, mice with renal IR were administered the IL-11 prior to and after renal injury, and histological analysis was performed.

Materials and Methods

Mice were treated as described in Example 2. Kidney (cortex and cortico-medullary junction) samples were collected after IR injury to examine the severity of renal dysfunction (renal tubular necrosis, apoptosis and neutrophil infiltration).

Morphological assessment of hematoxylin and eosin (H&E) staining was performed by an experienced renal pathologist, who was unaware of the treatment that each animal had received.

An established grading scale of necrotic injury (0-4, Renal Injury Score) to the proximal tubules was used for the histopathological assessment of IR-induced damage as outlined by Jablonski *et al.* (1983) and as described previously (Lee *et al.* (2007); Lee *et al.* (2004(1)).

Apoptosis after renal IR with TUNEL staining was detected as described in Park *et al.* (2010) using a commercially available *in situ* cell death detection kit (Roche, Indianapolis, IN) according to the instructions provided by the manufacturer.

Kidney inflammation after renal IR was assessed by detection of neutrophil infiltration using immunohistochemistry 24 hours after IR as described in Park *et al.* (2010). Neutrophils infiltrating the kidney were quantified in 5-7 randomly chosen 400X microscope images fields in the corticomedullary junction and results were expressed as neutrophils counted per 400X field. Apoptotic TUNEL positive cells were quantified in 5-7 randomly chosen 200X microscope images fields in the corticomedullary junction and results were expressed as neutrophils counted per 200X field.

Results

Figure 1A shows the severe necrotic renal injury in saline- or PEG-treated mice subjected to IR, 24 hours after injury. Compared to sham-operated vehicle-treated mice (not shown), the kidneys of vehicle-treated (saline or PEG) mice subjected to renal IR showed significant tubular necrosis, and proteinaceous casts with increased congestion (Figure 1A). Consistent with the plasma creatinine data, mice treated with HR IL-11 or PEGylated IL-11, 10 minutes before, or 30 minutes after renal ischemia, had reduced renal necrosis and tubular injury (Figure 1A).

The Jablonski scale (Jablonski *et al.* (1983)) renal injury score (graded from hematoxylin and eosin staining, scale: 0-4, with 0 being the least amount of renal injury and 4

being the most amount of renal injury) was used to grade renal tubular necrosis after thirty minutes of renal ischemia and 24 hours of reperfusion. This IR resulted in severe acute tubular necrosis (with renal injury scores approaching 4) in saline- or PEG-treated mice after IR. In contrast, mice treated with either HR IL-11 or PEGylated IL-11 before or after renal ischemia had partial, but significantly lower, renal injury scores compared to vehicle-treated mice subjected to renal IR (Table 3).

Table 3 - IL-11 and PEG IL-11 reduce Jablonski scale renal injury scores for mice subjected to renal IR

Data are means ± SEM. #P<0.05 vs. vehicle-treated mice subjected to renal IR.

Treatment	Renal Injury Score
Saline IR	3.75 ±0.25
PEG IR	3.75 ±0.14
HR IL-11 10 min before ischemia	1.75 ±0.48
HR IL-11 30 min after reperfusion	1.875 ±0.13
HR IL-11 60 min after reperfusion	2.5 ±0.29
PEG IL-11 10 min before ischemia	2.0 ±0.35
PEG IL-11 30 min after reperfusion	2.0 ±0.58
PEG IL-11 60 min after reperfusion	2.375 ±0.13

Renal ischemia and 24 hour of reperfusion resulted in severe apoptosis in the kidneys of saline- or PEG-treated mice. The TUNEL staining detected apoptotic renal cells in kidney of mice subjected to renal IR with predominant proximal tubule cell apoptosis (Figure 1B, magnification 200X). HR IL-11 or PEG IL-11 given before or after renal ischemia significantly reduced the number of apoptotic TUNEL-positive cells in the kidney (Figure 1C).

Figure 1D shows representative images of neutrophil immunohistochemistry of kidneys (magnification 400X) from mice subjected to 30 minutes of renal ischemia and 24 hour reperfusion. As shown by the dark brown color of the cells, there was significant neutrophil infiltration in the kidneys of mice treated with saline or PEG and subjected to 24 hour renal IR. In sham-operated mice, no neutrophils were found in the kidney (data not shown). Mice treated with HR IL-11 or PEG IL-11, before or after renal ischemia, had significantly reduced neutrophil infiltration in the kidney after IR (Figure 1E).

Saline or PEG-treated mice showed severe renal tubular necrosis, apoptosis, and neutrophil infiltration after IR, whereas pre- or post- IR HR IL-11 or PEGylated IR-11 treatment significantly attenuated renal IR injury in mice.

The data indicate that HR IL-11 or PEGylated IL-11 administration before or after renal IR injury protects against renal injury, as measured by a variety of histological criteria, including reduction in renal necrosis and renal tubular injury, reduction in numbers of apoptotic renal cells, and reduction in numbers of infiltrating neutrophils.

Example 4- IL-11 Reduces Necrosis and Apoptosis in Human Kidney Proximal Tubule Cells

In order to test whether HR IL-11 or PEGylated IL-11 administration protects against injury at the cellular level, human kidney proximal tubule cells with induced necrosis and apoptosis were administered the IL-11 prior to injury induction.

Materials and Methods

HK-2 cells, as described in Example 1C, were induced with necrotic injury by exposure to 5 mM H_2O_2 for 3 hours and lactate dehydrogenase (LDH) released into cell culture media was measured as described in Lee and Emala (2002) using a commercial LDH assay kit (Promega, Madison, WI).

To induce apoptosis, HK-2 cells were exposed to tumor necrosis factor (TNF)-α (20 ng/mL) plus cycloheximide (10 μg/mL) for 16 hours as described previously (Lee *et al.* (2007)). Cycloheximide was added in addition to TNF-α to facilitate apoptosis. Cycloheximide has been shown to synergistically increase TNF-α cytotoxicity (Ruff and Gifford (1981); Wright *et al.* 1992)).

HK-2 cell apoptosis was assessed by detecting poly-(adensosine diphosphate-ribose)-polymerase (PARP) and caspase 3 fragmentations on a Western Blot as previously described Lee *et al.* (2007).

Some HK-2 cells were pretreated with 10-1000 ng/mL HR IL-11 or PEGylated IL-11 (as described in Example 1A) 30 minutes before induction of necrosis or apoptosis.

Results

HK-2 cells pretreated with either HR IL-11 (data not shown) or PEGylated IL-11 (10-1000 ng/ml) (Table 4) for 30 minutes were protected against H_2O_2-induced necrosis, as evidenced by reduced LDH release, and apoptosis, as evidenced by reduced PARP and

caspase 3 fragmentation (Figure 2, 100 ng/mL PEGylated IL-11). HR IL-11 or PEGylated IL-11 treatments themselves had no effect on basal LDH released into the cell culture media.

Table 4 - PEGylated IL-11 reduces necrosis and apoptosis in human renal proximal tubule (HK-2) cells

Lactate dehydrogenase (LDH) released after H_2O_2-induced necrosis (5 mM H_2O_2 for 3 hours) in HK-2 cells (N=6 for each group, expressed as a percentage of total LDH released). Data are means ± SEM. *P<0.05 versus PEG-treated group.

Treatment	% LDH Released
PEG	26.82 ±1.92
PEG IL-11 (10 ng/mL)	17.74 ±1.93*
PEG IL-11 (100 ng/mL)	16.37 ±1.17*
PEG IL-11 (1000 ng/mL)	18.01 ±0.87*

Example 5- IL-11 Increases SK1 Synthesis and Induces SK Activity in HK-2 Cells

To determine if IL-11 induces SK1, a well-known mediator of cell protection, SK1 mRNA and protein were measured in HK-2 cells after IL-11 treatment.

Materials and Methods

Separate cohorts of HK-2 cells (Example 1C) were treated with 100 ng/mL HR IL-11 or PEGylated IL-11 (as described in Example 1A) for 6 hours to test for induction of SK1 or SK2 mRNA and for 16 hours to test for protein (N=4 per group).

mRNA encoding human SK1 or SK2 was measured 6 hours after HR IL-11 or PEGylated IL-11 treatment in HK-2 cells as described in Kim *et al.* (2010)(1). Table 5 lists the primer sequences utilized. HK-2 cell lysates were also collected for immunoblotting analyses of SK1, SK2 and β-actin (internal protein loading control) 16 hours after HR IL-11 or PEGylated IL-11 treatment as described previously (Kim *at al.* (2010)(1)).

SK activity was measured as described previously by Kim *et al.* (2007) using a modified protocol according to Vessey *et al.* (2005). To preferentially measure SK1 activity, the assay buffer was supplemented with 250 mM KCl plus 0.5% Triton X-100 (Klawitter *et al.* (2007); Pitman *et al.* (2012)).

Table 5 - Human RT-PCR primers

bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SK, sphingosine kinase. Respective anticipated RT-PCR product size, PCR cycle number for linear amplification and annealing temperatures used for each primer are also provided.

Primers	*Sequences (Sense/Antisense)*	*Product size (bp)*	*Cycle number*	*Annealing Temp (°C)*
SK1	*5'-ATCTCCTTCACGCTGATGC-3'* (SEQ ID NO: 1) *5'-GTGCAGAGACAGCAGGTTCA-3'* (SEQ ID NO: 2)	*330*	*26*	*66*
SK2	*5'-GGAGGAAGCTGTGAAGATGC-3'* (SEQ ID NO: 3) *5'-GCAGGTCAGACACAGAACGA-3'* (SEQ ID NO: 4)	*482*	*22*	*66*
GAPDH	*5'-ACCACAGTCCATGCCATCAC-3'* (SEQ ID NO: 5) *5'-CACCACCCTGTTGCTGTAGCC-3'* (SEQ ID NO: 6)	*450*	*16*	*65*

Results

Both HR IL-11 and PEGylated IL-11 (data not shown) increased SK1 mRNA and protein expression (Figure 3) and SK activity in HK-2 cells (Table 6). SK2 mRNA or protein expression did not change.

Table 6 - SK activity in HK-2 cells treated with saline or HR IL-11

Data are presented as means ± SEM. *P<0.05 vs. saline treatment.

Treatment	**HK-2 cell SK activity (pmol/mg protein/min)**
Saline	3.303 ±0.10
HR IL-11	6.265 ±0.63*

Example 6- Critical role of SK1 in IL-11-mediated renal protection against IR in mice

In order to determine if IL-11 requires the induction of SK1 for renal protection in mice, $SK1^{-/-}$ and $SK2^{-/-}$ mice subject to IR were treated with IL-11.

Materials and Methods

$SK1^{-/-}$ and $SK2^{-/-}$ mice described in Example 1B were treated with either 1 mg/kg HR IL-11, 1 mg/kg PEGylated IL-11, saline or PEG, and subjected to renal IR as described in Example 2.

Plasma samples were collected 24 hours after IR injury to examine the severity of renal dysfunction by measurements of plasma creatinine, as described in Example 2.

Results

$SK1^{-/-}$ mice were not protected against renal IR. $SK1^{-/-}$ mice subject to IR and treated with either 1 mg/kg HR IL-11 (Cr=2.95±0.15 mg/dL, N=4) or with 1 mg/kg PEGylated IL-11 (Cr=3.0±0.18 mg/dL, N=4) as compared to saline- (Cr=2.95±0.15 mg/dL, N=4) or PEG-treated (Cr=3.0±0.2 mg/dL, N=4), had the same impairment of kidney function as measured by plasma creatinine kinase.

In contrast, HR IL-11 (Cr=1.76±0.12 mg/dL, N=4) or PEGylated IL-11 (Cr=1.52±0.15 mg/dL, N=4) protected $SK2^{-/-}$ mice against renal IR as shown by the decrease in creatinine kinase.

Example 7- Hypoxia-inducible factor (HIF)-1α plays a critical role in IL-11 mediated SK1 induction

Since HIF-1α signaling can induce SK1 (Schwalm *et al.* (2008)), the hypothesis that IL-11 induces SK1 via a HIF-1α dependent mechanisms in HK-2 cells was tested.

Materials and Methods

HK-2 cells (Example 1C) were treated with HR IL-11 (100 ng/mL for 6 hours) or PEGylated IL-11 (100 ng/mL for 6 hours) (Example 1A).

To inhibit HIF-1α, other HK-2 cells (Example 1C) were pretreated with 10 μM 2-methoxyestradiol (2ME, a posttranscriptional down-regulator of HIF-1α) (Mabjeesh *et al.* (2003); Volpi *et al.* (2011)) or with 25 μM 3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1, an inhibitor of HIF-1α activity) (Hsu *et al.* (2003); Wu *et al.* (2003)) 30 min before HR IL-11 or PEGylated IL-11 treatment.

Nuclear extracts from HK-2 cells were prepared using the Transfactor Extraction Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. HIF-1α DNA-binding activity in nuclear extracts was determined using a TransFactor Family Colorimetric kit specific for HIF-1α (Clontech) according to the manufacturer's instructions.

PCR and mRNA quantification was performed as previously described in Kim *et al.* (2010) (1).

Results

HK-2 cells (N=4-6 for each group) were treated with saline (vehicle for HR IL-11), HR IL-11 (100 ng/mL for 6 hours) or PEGylated IL-11 (100 ng/mL for 6 hours). Treatment with HR IL-11 or PEG IL-11 increased nuclear HIF-1α DNA binding (Table 7). This result shows that IL-11 directly and significantly increases nuclear HIF-1α translocation and subsequent DNA binding in HK-2 cells.

Table 7 - IL-11 and PEG IL-11 induce nuclear translocation of HIF-1α in HK-2 cells. Data are means ± SEM. *P<0.05 vs. respective controls.

Treatment	**Relative HIF-1 alpha nuclear translocation (% saline control)**
Saline	100 ±0
PEG	103.5 ±4.5
HR IL-11	185 ±8.39*
PEG IL-11	207 ±11.59*

PEGylated IL-11 significantly and selectively increased SK1 mRNA expression in HK-2 cells without affecting SK-2 mRNA expression. However, when HK-2 cells were pretreated with inhibitors of HIF-1α signaling (2ME (10 μM) or YC-1 (25 μM)) 30 minutes before exposure to HR IL-11 (data not shown) or PEGylated IL-11 (100 ng/mL for 6 hours),

induction of SK1 mRNA was significantly attenuated without affecting SK2 mRNA expression (Table 8). Similar results were obtained by treating cells with HR IL-11.

Table 8- HIF-1α plays a critical role in IL-11 mediated SK1 induction

Densitometric quantification of relative SK1 or SK2 mRNA normalized to GAPDH from RT-PCR reactions in HK-2 cells treated with PEG or with 100 ng/mL PEGylated IL-11

*P<0.05 vs. PEG control group.

Treatment	SK1 mRNA/GAPDH over Vehicle	SK2 mRNA/GAPDH over Vehicle
PEG	1 ±0	1 ±0
PEG IL-11	1.8 ±0.12*	1.005 ±0.06
PEG + YC-1	0.85 ±0.05	0.98 ±0.08
PEG IL-11 + YC-1	0.972 ±0.13	0.972 ±0.13
PEG + 2ME	1.074 ±0.05	1.04 ±0.02
PEG IL-11 + 2ME	1.088 ±0.11	0.948 ±0.04

Example 8- The Hepatic Protective Effect of IL-11 Administration

In order to test whether HR IL-11 or PEGylated IL-11 administration before hepatic IR injury protects against damage, mice with hepatic IR were administered the IL-11 prior to hepatic injury, and tests for hepatic function performed.

Materials and Methods

Mice as described in Example 1B were pretreated with PEG (vehicle for PEGylated IL-11), HR IL-11 (1 mg/kg) or long acting PEGylated IL-11 (1 mg/kg) (both as described in Example 1A), 10 minutes prior to hepatic ischemia or sham-operation.

All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Male C57BL/6 mice (20-25 g, Harlan, Indianapolis, IN) were subjected to liver IR injury as described previously (Park *et al.* (2009)). This method of partial hepatic ischemia results in a segmental (approximately 70%) hepatic ischemia but spares the right lobe of the liver and prevents mesenteric venous congestion by allowing portal decompression through the right and caudate lobes of the liver. Sham operated mice were subjected to laparotomy and identical liver manipulations without the vascular occlusion.

Plasma samples were collected 5 and 24 hours after IR injury to examine the severity of hepatic dysfunction by measurements of plasma amino alanine transferase ("ALT") as a measure of liver injury using the Infinity™ ALT assay kit according to manufacturer's instructions (ThermoFisher Scientific, Waltham, MA.).

ALT is an enzyme found primarily in the liver, but also to a lesser extent in other tissues, such as kidney, heart, muscles, and pancreas. Blood normally contains low levels of ALT, but when the liver is damaged the liver releases ALT into the bloodstream. Thus, elevated plasma ALT levels are indicative of liver dysfunction or injury.

Results

Plasma ALT significantly increased in PEG-treated mice subjected to hepatic IR (ALT =19,942±1064 N=9 at 5 hours and 15,076±1174 N=25 at 24 hours) as compared to sham-operated mice (ALT=53+6 N=6 at 5 hours and ALT=61+8 N=8 at 24 hours). Pretreatment 10 minutes before hepatic ischemia with HR IL-11 (ALT=14,176±472 N=3 at 5 hours and 10,360±472 N=3 at 24 hours) or PEGylated IL-11 (designated "PEG-IL11") (ALT=14,358±949 N=3 at 5 hours and 10,541±1024 N=3 at 24 hours) partially but significantly attenuated the increases in plasma amino alanine transferase in mice (Table 9). These data indicate that treatment with HR IL-11 or PEGylated IL-11 protects the liver from IR injury.

Table 9 – Hepatic protection with pre-ischemic IL-11 treatment

Treatment	Time measured	ALT (U/L)
Sham	5 hours	53±6
Sham	24 hours	61±8
PEG IR	5 hours	19,942±1064
PEG IR	24 hours	15,076±1174
HR IL-11 (1 mg/kg) IR	5 hours	14,176±472
HR IL-11 (1 mg/kg) IR	24 hours	10,360±472
PEG IL-11 (1 mg/kg) IR	5 hours	14,358±949
PEG IL-11 (1 mg/kg) IR	24 hours	10,541±1024

Example 9- IL-11 reduces necrosis in human intestinal cells

In order to test whether HR IL-11 or PEGylated IL-11 administration protects against injury at the cellular level in the intestines, IEC-6 cells with induced necrosis were administered with the IL-11 prior to injury induction.

Materials and Methods

IEC-2 cells, as described in Example 1C, were induced with necrotic injury by exposure to 1 mM or 500 μM H_2O_2 for three hours and lactate dehydrogenase (LDH) released into cell culture media was measured as described in Lee and Emala (2002) using a commercial LDH assay kit (Promega, Madison, WI) at 2, 4, 6, and 8 hours.

Some IEC-6 cells were pretreated with 10-1000 ng/mL of HR IL-11 or PEGylated IL-11 (as described in Example 1A) for 30 minute prior to induction of necrosis.

Results

IEC-2 cells pretreated with either HR IL-11 (designated "WT IL-11") (Figures 4A and 4B) or PEGylated IL-11 (designated "PEG IL-11") (Figure 4A) for 30 minutes were protected against H_2O_2-induced necrosis as evidenced by reduced LDH release. These data indicate that treatment with HR IL-11 or PEGylated IL-11 will protect the intestine from IR injury.

References

Allende ML, Dreier JL, Mandala S and Proia RL. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *J Biol Chem* 279: 15396-15401, 2004 (1).

Allende ML, Sasaki T, Kawai H, Olivera A, Mi Y, Echten-Deckert G, Hajdu R, Rosenbach M, Keohane CA, Mandala S, Spiegel S and Proia RL. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J Biol Chem* 279: 52487-52492, 2004(2).

Allende ML, Yamashita T and Proia RL. G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood* 102: 3665-3667, 2003.

Bajwa A, Jo SK, Ye H, Huang L, Dondeti KR, Rosin DL, Haase VH, Macdonald TL, Lynch KR and Okusa MD. Activation of sphingosine-1-phosphate 1 receptor in the proximal tubule protects against ischemia-reperfusion injury. *J Am Soc Nephrol* 21: 955-965, 2010.

Bonventre JV and Weinberg JM. Recent advances in the pathophysiology of ischemic acute renal failure. *J Am Soc Nephrol* 14: 2199-2210, 2003.

Bozza M, Bliss JL, Maylor R, Erickson J, Donnelly L, Bouchard P, Dorner AJ and Trepicchio WL. Interleukin-11 reduces T-cell-dependent experimental liver injury in mice. *Hepatology* 30: 1441-1447, 1999.

Chae SS, Proia RL and Hla T. Constitutive expression of the S1P1 receptor in adult tissues. *Prostaglandins Other Lipid Mediat* 73: 141-150, 2004.

Cleland JL, Geething NC, Moore JA, Rogers BC, Spink BJ, Wang C-W, Alters SE, Stemmer WP and Schellenber V. A novel long-acting human growth hormone fusion protein (VRS-317): enhanced in vivo potency and half-life. *J Pharmaceutical Sciences* 101: 2744-2754, 2012.

Cox GN, Rosendahl MS, Chlipala EA, Smith DJ, Carlson SJ and Doherty DH. A long-acting, mono-PEGylated human growth hormone analog is a potent stimulator of weight gain and bone growth in hypophysectomized rats. *Endocrinology* 148: 1590-1597, 2007.

Du X and Williams DA. Interleukin-11: review of molecular, cell biology, and clinical use. *Blood* 89: 3897-3908, 1997.

Faubel S. Acute kidney injury and multiple organ dysfunction sindrome. *Minerva Urol Nefrol* 61: 171-188, 2009.

Fujio Y, Maeda M, Mohri T, Obana M, Iwakura T, Hayama A, Yamashita T, Nakayama H and Azuma J. Glycoprotein 130 Cytokine Signal as a Therapeutic Target Against Cardiovascular Diseases. *J Pharmacol Sci* 117:213-222 2011.

Goldman SC, Bracho F, Davenport V, Slack R, Areman E, Shen V, Lenarsky C, Weinthal J, Hughes R and Cairo MS. Feasibility study of IL-11 and granulocyte colony-stimulating factor after myelosuppressive chemotherapy to mobilize

peripheral blood stem cells from heavily pretreated patients. *J Pediatr Hematol Oncol* 23: 300-305, 2001.

Grosfeld JL, Du X and Williams DA. Interleukin-11: its biology and prospects for clinical use. *JPEN J Parenter Enteral Nutr* 23: S67-S69, 1999.

Hait NC, Oskeritzian CA, Paugh SW, Milstien S and Spiegel S. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. *Biochim Biophys Acta* 1758: 2016-2026, 2006.

Hoste EA and Kellum JA. Incidence, classification, and outcomes of acute kidney injury. *Contrib Nephrol* 156: 32-38, 2007.

Hsu HK, Juan SH, Ho PY, Liang YC, Lin CH, Teng CM and Lee WS. YC-1 inhibits proliferation of human vascular endothelial cells through a cyclic GMP-independent pathway. *Biochem Pharmacol* 66: 263-271, 2003.

Ikeda M, Prachasilchai W, Burne-Taney MJ, Rabb H and Yokota-Ikeda N. Ischemic acute tubular necrosis models and drug discovery: a focus on cellular inflammation. *Drug Discov Today* 11: 364-370, 2006.

Jablonski P, Howden BO, Rae DA, Birrel CS, Marshall VC and Tange J. An experimental model for assessment of renal recovery from warm ischemia. *Transplantation* 35: 198-204, 1983.

Jo SK, Bajwa A, Ye H, Vergis AL, Awad AS, Kharel Y, Lynch KR and Okusa MD. Divergent roles of sphingosine kinases in kidney ischemia-reperfusion injury. *Kidney Int* 75: 167-175, 2009.

Jo SK, Rosner MH and Okusa MD. Pharmacologic treatment of acute kidney injury: Why drugs haven't worked and what is on the horizon. *Clin J Am Soc Nephrol* 2: 356-365, 2007.

Jones DR and Lee HT. Perioperative renal protection. *Best Pract Res Clin Anaesthesiol* 22: 193-208, 2008.

Kim M, Chen SW, Park SW, Kim M, D'Agati VD, Yang J and Lee HT. Kidney-specific reconstitution of the A1 adenosine receptor in A1 adenosine receptor knockout mice reduces renal ischemia-reperfusion injury. *Kidney Int* 75: 809-823, 2009.

Kim M, Kim M, Kim N, D'Agati VD, Emala CW, Sr. and Lee HT. Isoflurane mediates protection from renal ischemia-reperfusion injury via sphingosine kinase and sphingosine-1-phosphate-dependent pathways. *Am J Physiol Renal Physiol* 293: F1827-F1835, 2007.

Kim M, Kim M, Park SW, Pitson SM and Lee HT. Isoflurane protects human kidney proximal tubule cells against necrosis via sphingosine kinase and sphingosine-1-phosphate generation. *Am J Nephrol* 31: 353-362, 2010 (1).

. **Kim M, Park SW, Kim M, Chen SW, Gerthoffer WT, D'Agati VD and Lee HT**. Selective Renal Over-Expression of Human Heat Shock Protein 27 Reduces Renal Ischemia-Reperfusion Injury in Mice. *Am J Physiol Renal Physiol* 2010 (2).

Kimura R, Maeda M, Arita A, Oshima Y, Obana M, Ito T, Yamamoto Y, Mohri T, Kishimoto T, Kawase I, Fujio Y and Azuma J. Identification of cardiac myocytes as the target of interleukin 11, a cardioprotective cytokine. *Cytokine* 38: 107-115, 2007.

Klawitter S, Hofmann LP, Pfeilschifter J and Huwiler A. Extracellular nucleotides induce migration of renal mesangial cells by upregulating sphingosine kinase-1 expression and activity. *Br J Pharmacol* 150: 271-280, 2007.

Kuenzler KA, Pearson PY and Schwartz MZ. IL-11 pretreatment reduces cell death after intestinal ischemia-reperfusion. *J Surg Res* 108: 268-272, 2002.

Lai PC, Cook HT, Smith J, Keith JC, Jr., Pusey CD and Tam FW. Interleukin-11 attenuates nephrotoxic nephritis in Wistar Kyoto rats. *J Am Soc Nephrol* 12: 2310-2320, 2001.

Leclercq TM and Pitson SM. Cellular signalling by sphingosine kinase and sphingosine 1-phosphate. *IUBMB Life* 58: 467-472, 2006.

Lee HT and Emala CW. Adenosine attenuates oxidant injury in human kidney proximal tubular cells via A1 and A2a adenosine receptor activation . *Am J Physiol Renal Physiol* 282(5): F844-F852, 2002.

Lee HT, Gallos G, Nasr SH and Emala CW. A1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice. *J Am Soc Nephrol* 15: 102-111, 2004(1).

Lee HT, Kim M, Jan M, Penn RB and Emala CW. Renal tubule necrosis and apoptosis modulation by A1 adenosine receptor expression. *Kidney Int* 71(12): 1249-1261, 2007.

Lee HT, Xu H, Nasr SH, Schnermann J and Emala CW. A1 adenosine receptor knockout mice exhibit increased renal injury following ischemia and reperfusion. *Am J Physiol Renal Physiol* 286: F298-F306, 2004(2).

Liu H, Chakravarty D, Maceyka M, Milstien S and Spiegel S. Sphingosine kinases: a novel family of lipid kinases. *Prog Nucleic Acid Res Mol Biol* 71: 493-511, 2002.

Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW and Giannakakou P. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell* 3: 363-375, 2003.

Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S and Proia RL. Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol* 25: 11113-11121, 2005.

Mooberry SL. Mechanism of action of 2-methoxyestradiol: new developments. *Drug Resist Updat* 6: 355-361, 2003.

Naugler KM, Baer KA and Ropeleski MJ. Interleukin-11 antagonizes Fas ligand-mediated apoptosis in IEC-18 intestinal epithelial crypt cells: role of MEK and Akt-dependent signaling. *Am J Physiol Gastrointest Liver Physiol* 294: G728-G737, 2008.

Obana M, Maeda M, Takeda K, Hayama A, Mohri T, Yamashita T, Nakaoka Y, Komuro I, Takeda K, Matsumiya G, Azuma J and Fujio Y. Therapeutic activation of signal transducer and activator of transcription 3 by interleukin-11 ameliorates cardiac fibrosis after myocardial infarction. *Circulation* 121: 684-691, 2010.

Park SW, Kim M, Chen SW, Kim M, D'Agati VD and Lee HT. Human activated protein C attenuates both hepatic and renal injury caused by hepatic ischemia and reperfusion injury in mice. *Kidney Int* 76: 739-750 2009

Park SW, Kim M, Chen SW, Brown KM, D'Agati VD and Lee HT. Sphinganine-1-phosphate protects kidney and liver after hepatic ischemia and reperfusion in mice through S1P(1) receptor activation. *Lab Invest* 90: 1209-1224, 2010.

Paul SR, Bennett F, Calvetti JA, Kelleher K, Wood CR, O'Hara RM Jr, Leary AC, **Sibley B, Clark SC, Williams DA and Yang Y-C.** Molecular cloning of a cDNA encoding interleukin-11, a stronal cell-line-derived lymphopoietic and hematopoietic cytokine. *Proc Natl Acad Sci USA* 87: 7512-7516, 1990.

Pitman MR, Pham DH and Pitson SM. Isoform-selective assays for sphingosine kinase activity. *Methods Mol Biol* 874: 21-31, 2012.

Putoczki T and Ernst M. More than a sidekick: the IL-6 family cytokine IL-11 links inflammation to cancer. *J Leukoc Biol* 88: 1109-1117, 2010.

Reynolds CH. Clinical efficacy of rhIL-11. *Oncology (Williston Park)* 14: 32-40, 2000.

Ropeleski MJ, Tang J, Walsh-Reitz MM, Musch MW and Chang EB. Interleukin-11-induced heat shock protein 25 confers intestinal epithelial-specific cytoprotection from oxidant stress. *Gastroenterology* 124: 1358-1368, 2003.

Rosenberger C, Rosen S, Shina A, Frei U, Eckardt KU, Flippin LA, Arend M, Klaus SJ and Heyman SN. Activation of hypoxia-inducible factors ameliorates hypoxic distal tubular injury in the isolated perfused rat kidney. *Nephrol Dial Transplant* 23: 3472-3478, 2008.

Ruff MR and Gifford GE. Rabbit tumor necrosis factor: mechanism of action. *Infect Immun* 31: 380-385, 1981.

Schellenberger V, Wang C-W, Geething NC, Spink BJ, Campbell A, To W, Scholle MD, Yin Y, Yao Y, Bogin O, Cleland JL, Silverman J, Stemmer WP. A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. *Nat Biotechnology* 27: 1186-1190, 2009.

Schodel J, Klanke B, Weidemann A, Buchholz B, Bernhardt W, Bertog M, Amann K, Korbmacher C, Wiesener M, Warnecke C, Kurtz A, Eckardt KU and Willam C. HIF-prolyl hydroxylases in the rat kidney: physiologic expression patterns and regulation in acute kidney injury. *Am J Pathol* 174: 1663-1674, 2009.

Schwalm S, Doll F, Romer I, Bubnova S, Pfeilschifter J and Huwiler A. Sphingosine kinase-1 is a hypoxia-regulated gene that stimulates migration of human endothelial cells. *Biochem Biophys Res Commun* 368: 1020-1025, 2008.

Sheridan BC, Dinarello CA, Meldrum DR, Fullerton DA, Selzman CH and McIntyre RC, Jr. Interleukin-11 attenuates pulmonary inflammation and vasomotor dysfunction in endotoxin-induced lung injury. *Am J Physiol* 277: L861-L867, 1999.

SLOT C. Plasma creatinine determination. A new and specific Jaffe reaction method. *Scand J Clin Lab Invest* 17: 381-387, 1965.

Smith JW. Tolerability and side-effect profile of rhIL-11. *Oncology (Williston Park)* 14: 41-47, 2000.

Takagi A, Yamashita N, Yoshioka T, Takaishi Y, Sano K, Yamaguchi H, Maeda A, Saito K, Takakura Y and Hashida M. Enhanced pharmacological activity of recombinant human interleukin-11 (rhIL11) by chemical modification with polyethylene glycol. *J Control Release* 119: 271-278, 2007.

Trepicchio WL, Bozza M, Pedneault G and Dorner AJ. Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production. *J Immunol* 157: 3627-3634, 1996.

Trepicchio WL and Dorner AJ. The therapeutic utility of Interleukin-11 in the treatment of inflammatory disease. *Expert Opin Investig Drugs* 7: 1501-1504, 1998.

Trepicchio WL, Wang L, Bozza M and Dorner AJ. IL-11 regulates macrophage effector function through the inhibition of nuclear factor-kappaB. *J Immunol* 159: 5661-5670, 1997.

Venkataraman K, Thangada S, Michaud J, Oo ML, Ai Y, Lee YM, Wu M, Parikh NS, Khan F, Proia RL and Hla T. Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. *Biochem J* 397: 461-471, 2006.

Vessey DA, Kelley M and Karliner JS. A rapid radioassay for sphingosine kinase. *Anal Biochem* 337: 136-142, 2005.

Vessey DA, Kelley M, Li L, Huang Y, Zhou HZ, Zhu BQ and Karliner JS. Role of sphingosine kinase activity in protection of heart against ischemia reperfusion injury. *Med Sci Monit* 12: BR318-BR324, 2006.

Volpi G, Facchinetti F, Moretto N, Civelli M and Patacchini R. Cigarette smoke and alpha,beta-unsaturated aldehydes elicit VEGF release through the p38 MAPK pathway in human airway smooth muscle cells and lung fibroblasts. *Br J Pharmacol* 163: 649-661, 2011.

Wadgaonkar R, Patel V, Grinkina N, Romano C, Liu J, Zhao Y, Sammani S, Garcia JG and Natarajan V. Differential regulation of sphingosine kinases 1 and 2 in lung injury. *Am J Physiol Lung Cell Mol Physiol* 296: L603-L613, 2009.

Waxman AB, Mahboubi K, Knickelbein RG, Mantell LL, Manzo N, Pober JS and Elias JA. Interleukin-11 and interleukin-6 protect cultured human endothelial cells from H2O2-induced cell death. *Am J Respir Cell Mol Biol* 29: 513-522, 2003.

Wright SC, Kumar P, Tam AW, Shen N, Varma M and Larrick JW. Apoptosis and DNA fragmentation precede TNF-induced cytolysis in U937 cells. *J Cell Biochem* 48: 344-355, 1992.

Wu Y, Dong Y, Song P and Zou MH. Activation of the AMP-activated protein kinase (AMPK) by nitrated lipids in endothelial cells. *PLoS One* 7: e31056, 2012.

Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS and Park JW. YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. *J Natl Cancer Inst* 95: 516-525, 2003.

CLAIMS

1. A method of preventing or treating an injury to an organ caused by ischemia, followed by reperfusion, comprising administering, to a subject in need thereof, a therapeutically effective amount of IL-11.

2. The method of claim 1, wherein the ischemia followed by reperfusion is the result of a surgical procedure.

3. The method of claim 2, wherein the IL-11 is administered prior to the surgical procedure.

4. The method of claim 3, wherein the IL-11 is administered within about one day or less prior to the surgical procedure.

5. The method of claim 3, wherein the IL-11 is administered within about six hours or less prior to the surgical procedure.

6. The method of claim 3, wherein the IL-11 is administered within about sixty minutes or less prior to the surgical procedure.

7. The method of claim 3, wherein the IL-11 is administered within about thirty minutes or less prior to the surgical procedure.

8. The method of claim 2, wherein the IL-11 is administered during the surgical procedure.

9. The method of claim 2, wherein the IL-11 is administered following the surgical procedure.

10. The method of claim 9, wherein the IL-11 is administered within about one day or less following the surgical procedure.

11. The method of claim 9, wherein the IL-11 is administered within about six hours or less following the surgical procedure.

12. The method of claim 9, wherein the IL-11 is administered within about sixty minutes or less following the surgical procedure.

13. The method of claim 9, wherein the IL-11 is administered within about thirty minutes or less following to the surgical procedure.

14. The method of claim 1, wherein the ischemia followed by reperfusion is the result of an event selected from the group consisting of: cardiopulmonary bypass during surgery, coronary bypass, major vascular repair, liver resection, organ transplantation, stroke, liver ischemia, kidney ischemia, aortic occlusion, myocardial occlusion, cardiac arrest, shock or trauma.

15. The method of claim 14, wherein the IL-11 is administered within about one day or less following the event.

16. The method of claim 14, wherein the IL-11 is administered within about six hours or less following the event.

17. The method of claim 14, wherein the IL-11 is administered within about sixty minutes or less following the event.

18. The method of claim 14, wherein the IL-11 is administered within about thirty minutes or less following to the event.

19. The method of claim 2, wherein the surgical procedure relates to an organ selected from the group consisting of: the heart, lungs, liver, kidneys, intestines or aorta.

20. The method of claim 1, wherein the IL-11 is native, recombinant or a variant thereof.

21. The method of claim 1, wherein the IL-11 is native, recombinant, or a variant thereof which is modified to increase the half life of the IL-11.

22. The method of claim 1, wherein the IL-11 is native, recombinant, or a variant thereof which is modified with polyethylene glycol.

23. The method of claim 1, wherein the IL-11 is a variant wherein a cysteine residue is added following the last amino acid of the IL-11 protein.

24. The method of claim 23, wherein the variant is modified with a polyethylene glycol at the added cysteine residue.

25. The method of claim 1, wherein the organ is selected from the group consisting of: a kidney, liver, lung, heart, blood vessel, aorta, or intestine.

26. The method of claim 1, wherein the subject is a mammal.

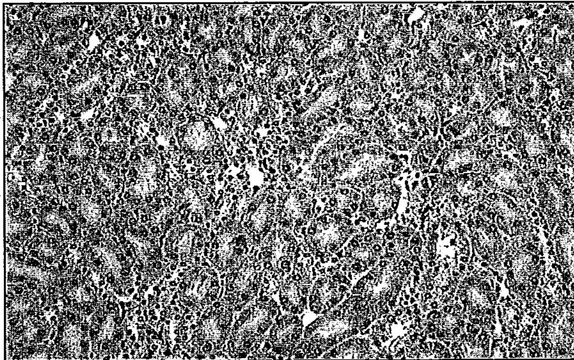
27. The method of claim 1, wherein the subject is a human.

28. The method of claim 1, wherein the IL-11 is administered following reperfusion of the ischemic organ.

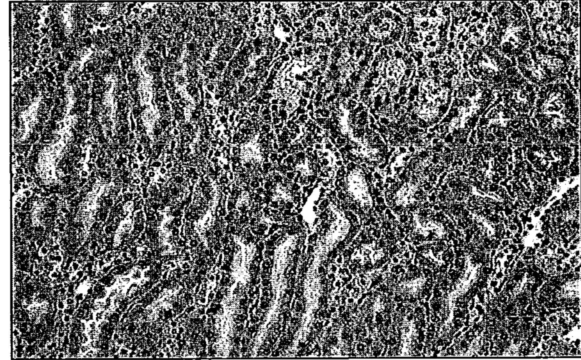
29. The method of claim 1, wherein the IL-11 is administered during reperfusion of the ischemic organ.

30. The method of claim 1, wherein the IL-11 is administered at the start of the reperfusion of the ischemic organ.

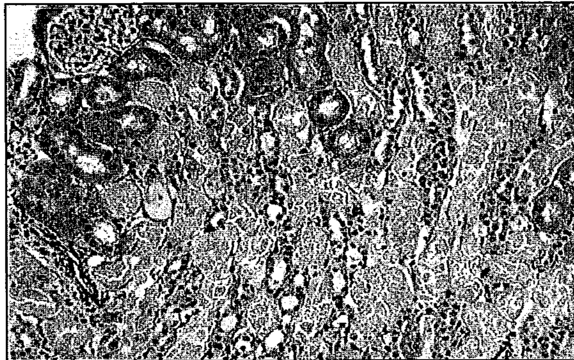
Figure 1A H&E (200X)



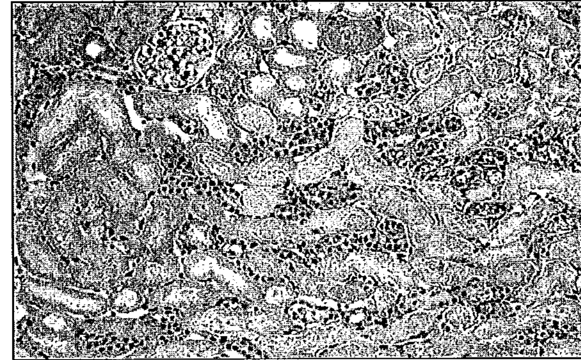
Saline sham



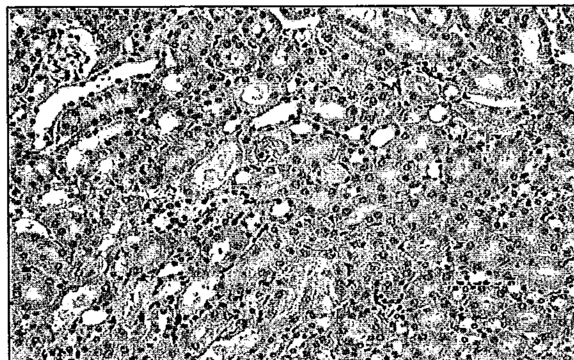
HR IL-11 sham



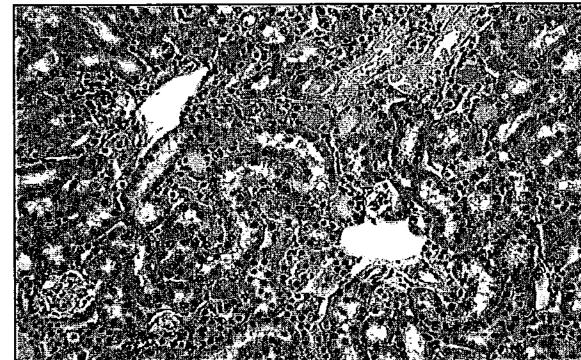
Saline IR



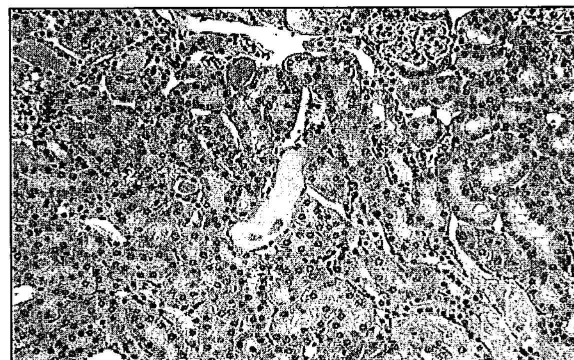
PEG IR



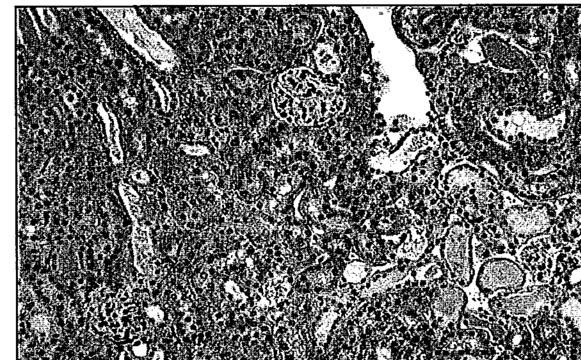
HR IL-11 10 min before ischemia IR



PEG IL-11 10 min before ischemia IR



HR IL-11 30 min after ischemia IR

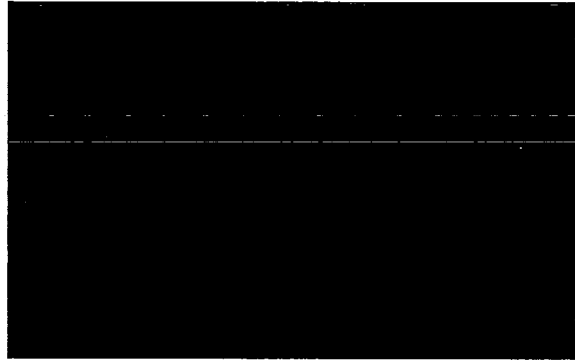


PEG IL-11 30 min after ischemia IR

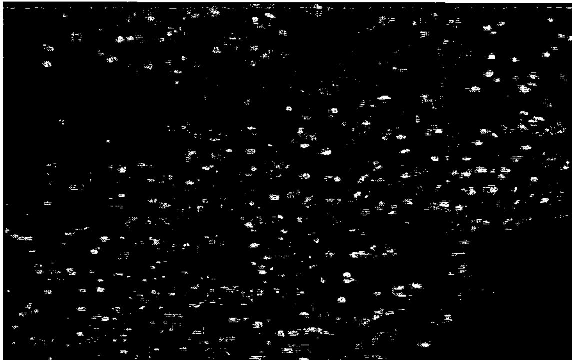
Figure 1B

TUNEL (200X)

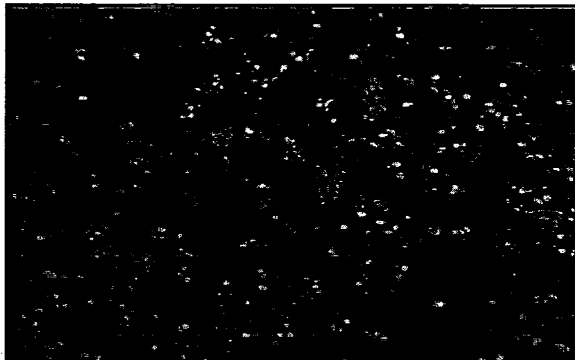
Saline sham



HR IL-11 sham



Saline IR



PEG IR

HR IL-11 10 min before ischemia IR

PEG IL-11 10 min before ischemia IR

HR IL-11 30 min after ischemia IR

PEG IL-11 30 min after ischemia IR

Figure 1C

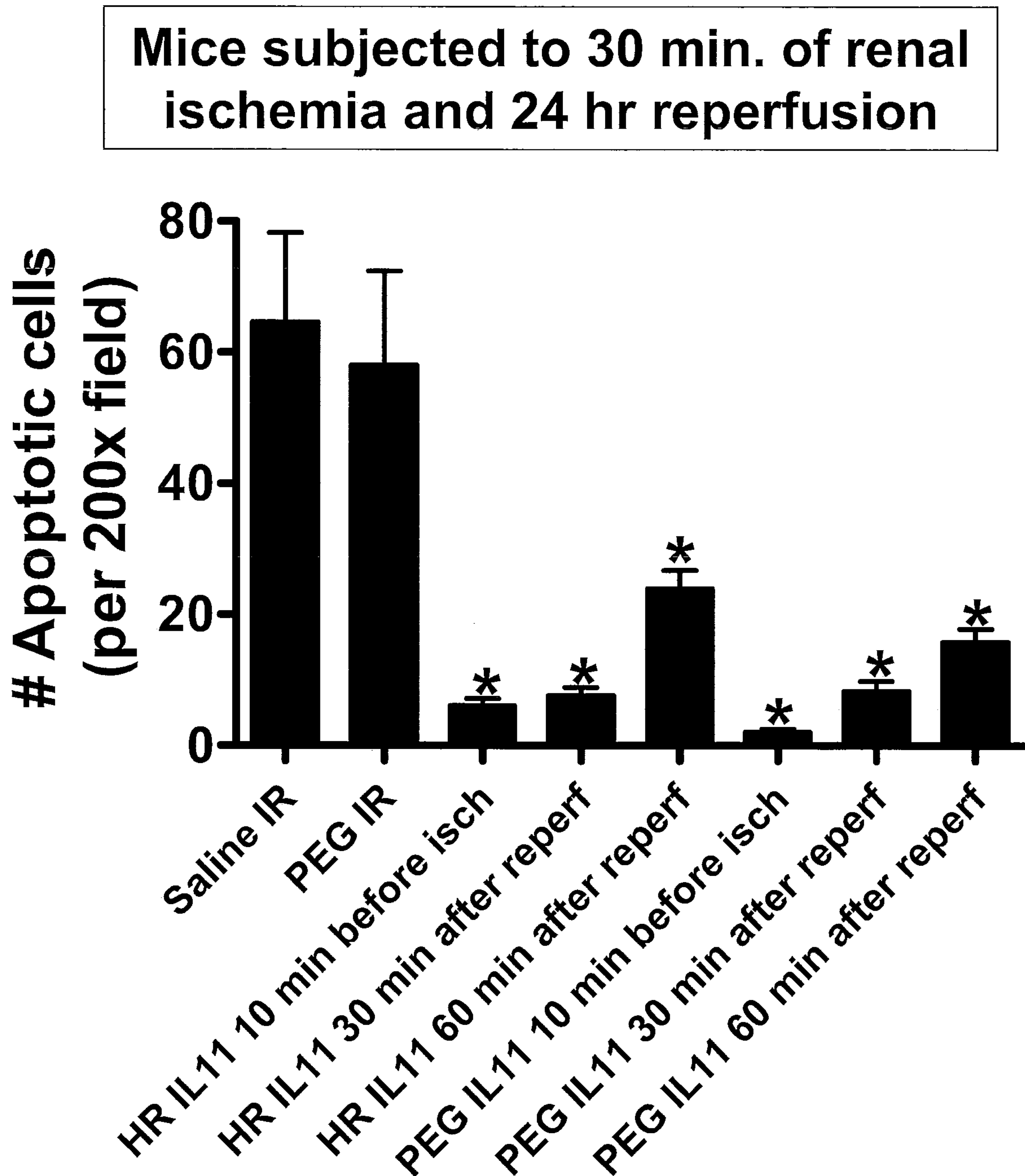
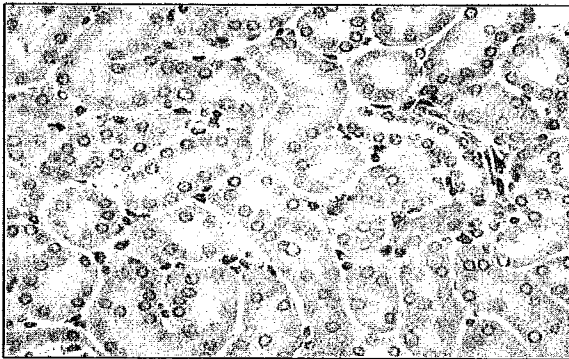
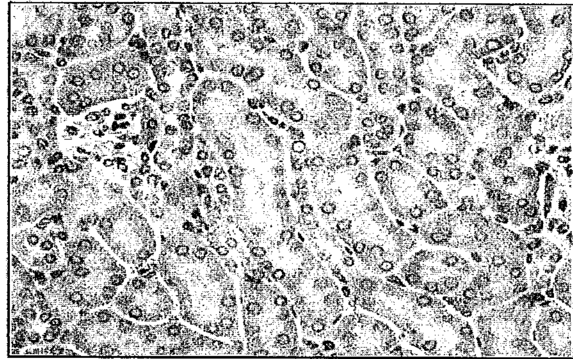
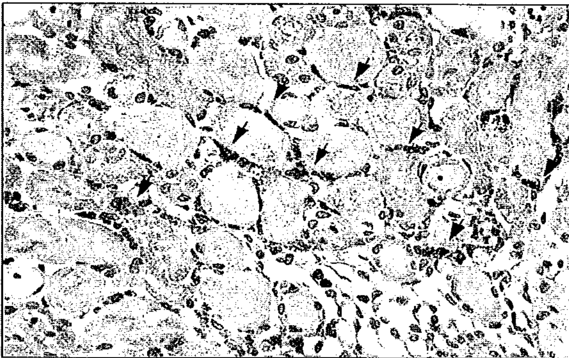
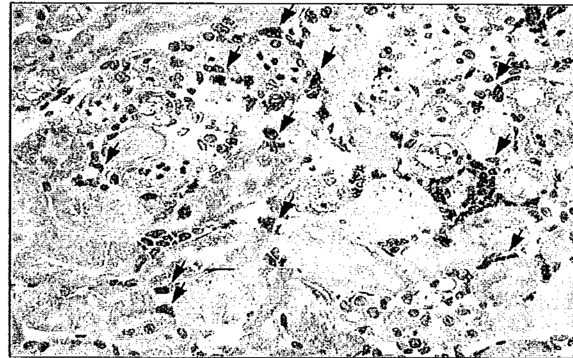


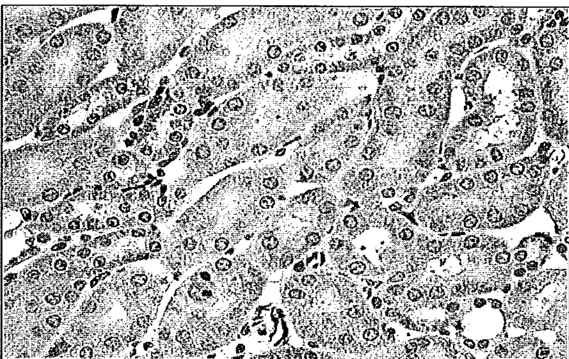
Figure 1D PMN (400X)

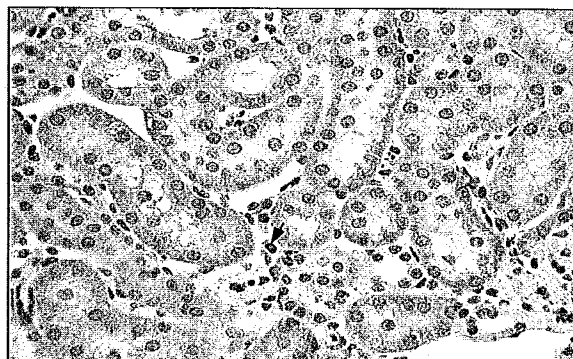

Saline sham

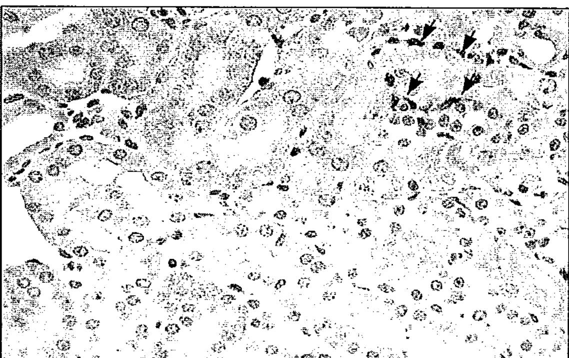

HR IL-11 sham


Saline IR


PEG IR


HR IL-11 10 min before ischemia IR


PEG IL-11 10 min before ischemia IR


HR IL-11 30 min after ischemia IR

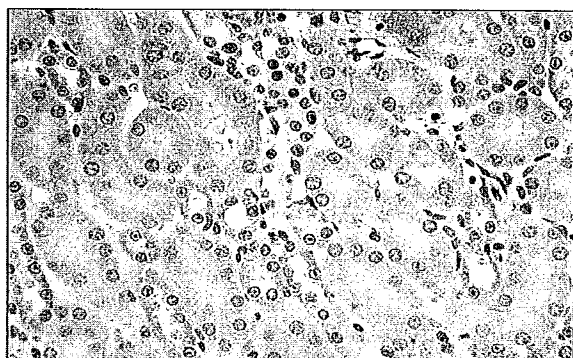

PEG IL-11 30 min after ischemia IR

Figure 1E

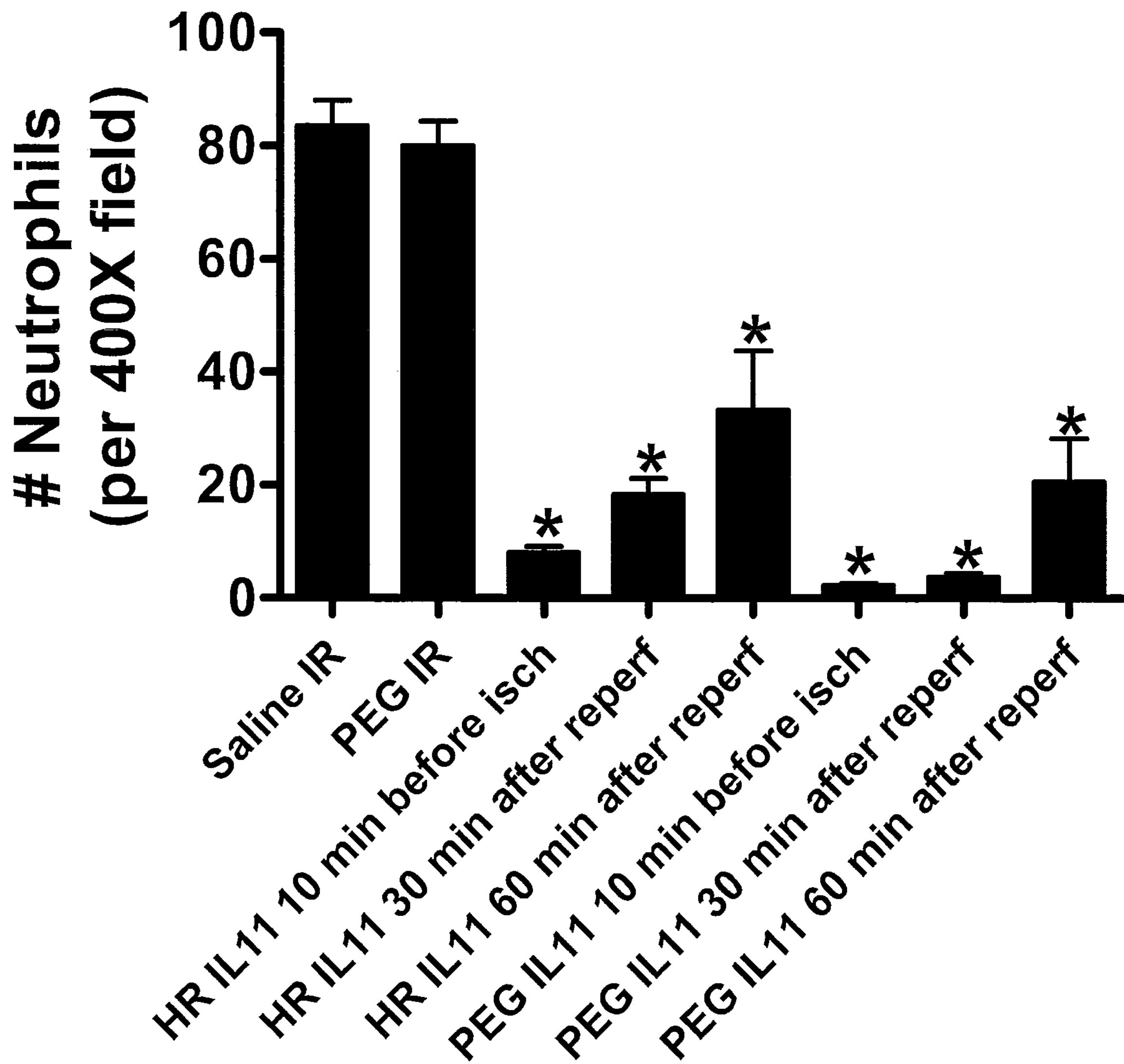


Figure 2

HK-2 cell apoptosis

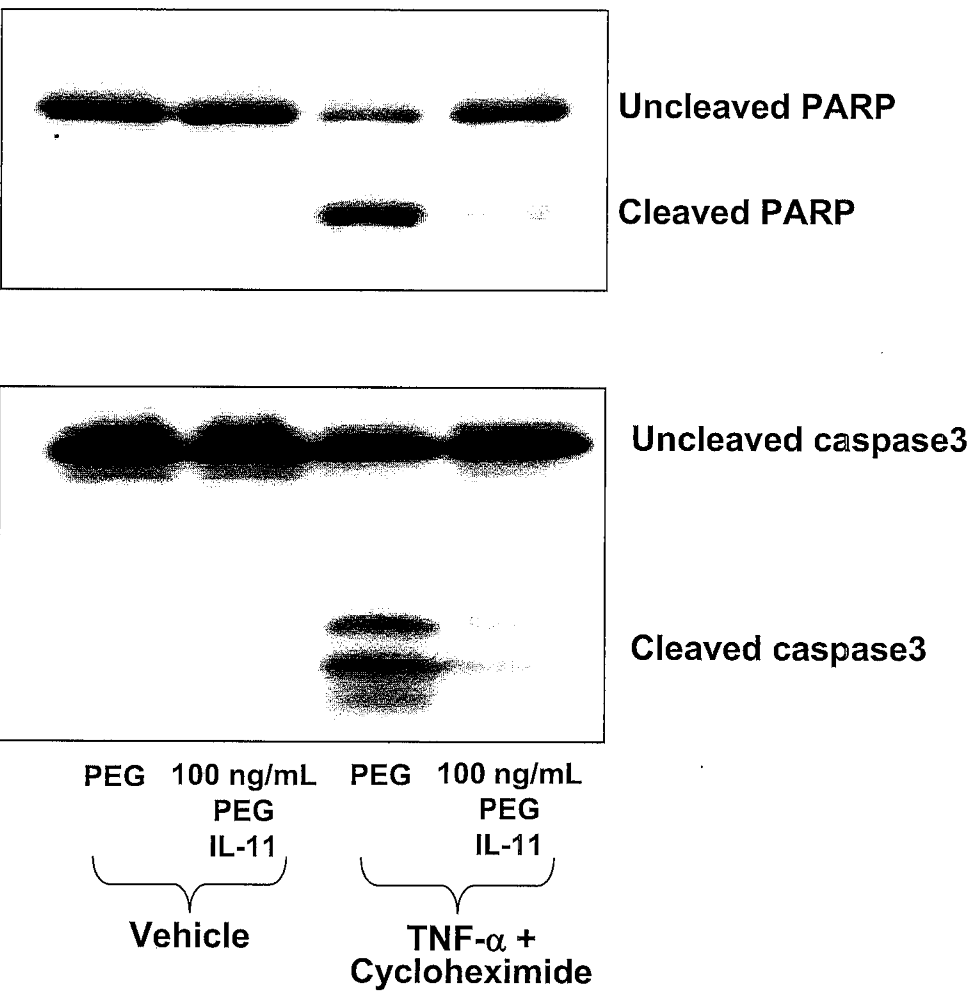


Figure 3

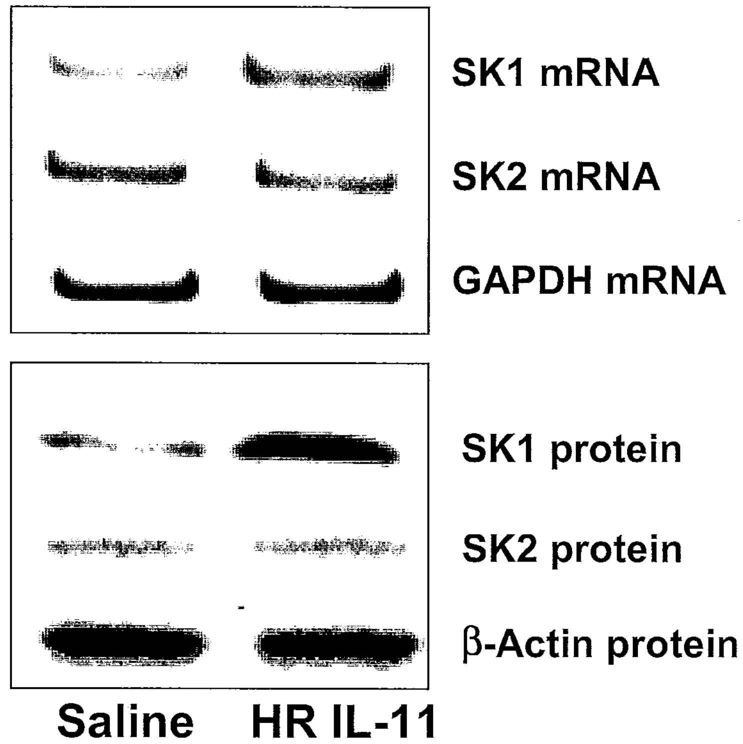


Figure 4

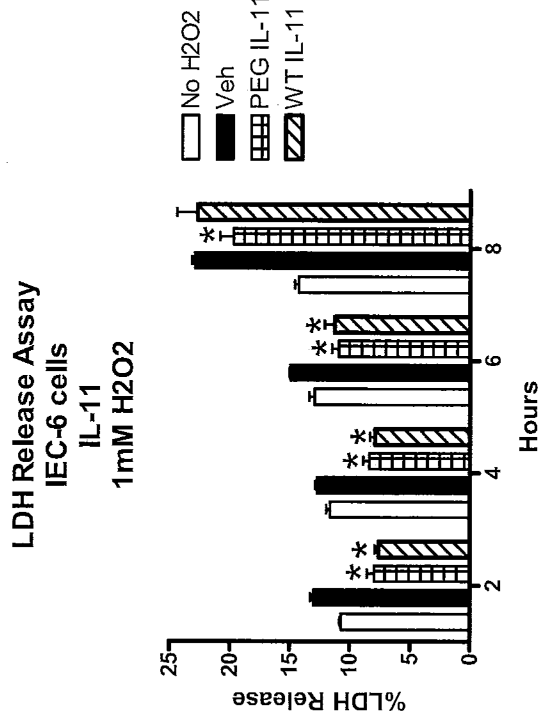


Figure 4A

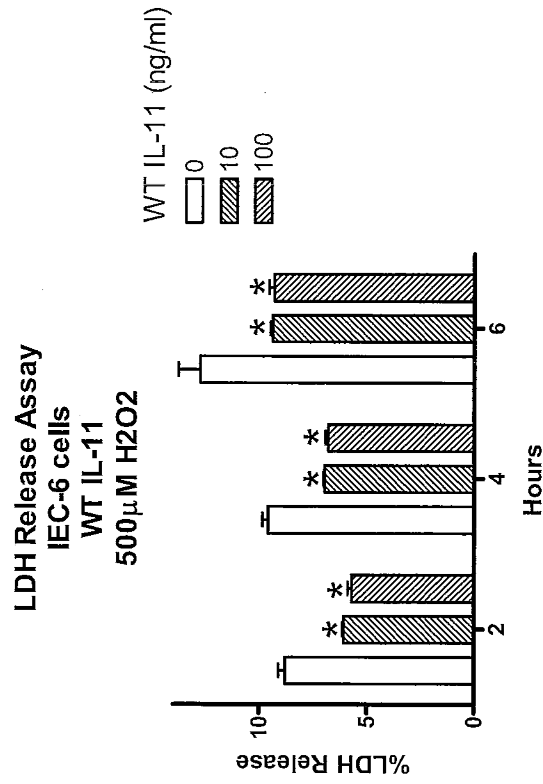


Figure 4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/49039

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 45/00; C07K 14/54 (2013.01)
USPC - 435/69.52; 530/402; 424/85.2
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(8): A61K 45/00, 38/40; C07K 14/54 (2013.01)
USPC: 435/69.52; 514/21.2; 530/402; 424/85.2, 85.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; Pubmed; ProQuest; ischemia, reperfusion, 'ischemic injury,' 'interleukin-11,' 'IL-11,' 'modified IL-11'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	KUENZLER, K et al. IL-11 Pretreatment Reduces Cell DeathAafter Intestinal Ischemia-Reperfusion. Journal of Surgical Research. December 2002, Vol. 108, No. 2, pp 268-272; page 271 right column, third paragraph to page 271, left column, first and second paragraph; page 269, left column, third paragraph. DOI: 10.1006/jsre.2002.6542.	1-3, 14, 19, 20, 25-27 ----- 4-13, 15-18, 21-24, 28-30
Y	US 2011/0229433 A1 (KUNGLE, A et al.) September 22, 2011; paragraph [0035]	4, 9-13, 15
Y	LANE, J et al. Interleukin-10 Reduces The Systemic Inflammatory Response In A Murine Model Of Intestinal Ischemia/Reperfusion. Surgery. August 1997, Vol. 122, No. 2, pp 288-294; page 290, first paragraph.	11, 12, 16, 17
Y	US 8133480 B2 (COX, G) March 13, 2012; column 18, lines 60-64; column 3, lines 14-18; Claims 1, 3	21-24
Y	BROWN, JM et al. Interleukin 1 Pretreatment Decreases Ischemia/Reperfusion Injury. Procedings of the National Academy of Science. July 1990, Vol. 87, No. 13, pp 5026-5030; page 5026, left column second paragraph to right column, first paragraph.	5-7
Y	AITSEBAOMO, J et al. Recombinant Human Interleukin-11 Treatment Enhances Collateral Vessel Growth After Femoral Artery Ligation. Arterioscler Thromb Vasc Biol. 11 November 2010, Vol. 31, pp 306-312; abstract; supplemental page 3, first paragraph. DOI: 10.1161/ATVBAHA.110.216986.	8, 28-30
Y	US 8071532 B2 (MANNESSE, M et al.) December 6, 2011; Claim 19	13, 18

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

* 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
25 November 2013 (25.11.2013)	03 DEC 2013

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774