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RECOMBINANT HUMAN MITSUGUMIN-53 COMPOSITIONS AND METHODS OF USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to, and the benefit of, U.S. Provisional Patent Application No. 63/517,479, filed August 3, 2023, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

10 This invention was made with government support under Grant Nos. HL143000 and DK123475 awarded by the National Institutes of Health. The government has certain rights in the invention.

REFERENCE TO SEQUENCE LISTING

15 The sequence listing submitted on August 2, 2024, as an .XML file entitled "103361-574PV1-ST26" created on July 30, 2024, and having a file size of 4,761 bytes is hereby incorporated by reference pursuant to 37 C.F.R. § 1.52(e)(5).

FIELD

20 The present disclosure relates to compositions and methods comprising recombinant human Mitsugumin-53 (rhMG53) and uses thereof.

BACKGROUND

25 Solid organ transplantation is plagued with ischemia reperfusion injury (IRI) which significantly negatively impacts early organ function. This early organ injury (also known as dysfunction, primary graft dysfunction (PGD), primary non-function) leads to poor short-term outcomes and has a role in chronic graft dysfunction or rejection leading to long-term worse outcomes. The endothelial cells are the barrier between the donor graft and the recipient's blood. The IRI leads to injury to the donor graft with the endothelial cells being initially susceptible with injury leading to cell death through apoptosis, necroptosis, and pyroptosis. This initial injury
30 allows for increased injury signaling which promotes recipient leukocyte migration into the donor graft leading to an increased IRI response.

 What is needed are new compositions and/or methods to prevent and treat ischemic reperfusion injury and/or primary graft dysfunction.

SUMMARY

Disclosed herein is a composition comprising recombinant human Mitsugumin-53 (rhMG53) protein and its use thereof in treating ischemia reperfusion injury (IRI) and/or primary graft dysfunction (PGD).

5 Accordingly, in some aspects, disclosed herein a perfusate composition comprising recombinant human Mitsugumin-53 (rhMG53) protein and a perfusion medium. In some embodiments, the rhMG53 comprises SEQ ID NO: 1.

In one aspect, disclosed herein is a preservation solution comprising recombinant human Mitsugumin-53 (rhMG53) protein and a preservation medium. In some embodiments, the rhMG53
10 comprises SEQ ID NO: 1.

In one aspect, disclosed herein is a method of treating or preventing ischemia reperfusion injury (IRI) in an *ex vivo* graft comprising administering a recombinant human Mitsugumin-53 (rhMG53) protein to the *ex vivo* graft.

In some embodiments, the rhMG53 protein is administered in a perfusion medium. In some
15 embodiments, the rhMG53 protein is administered in a preservation medium.

In some embodiments, the rhMG53 comprises SEQ ID NO: 1.

In some embodiments, the rhMG53 protein is administered to the *ex vivo* graft before reperfusion of the *ex vivo* graft. In some embodiments, the rhMG53 protein is administered to the *ex vivo* graft during reperfusion of the *ex vivo* graft. In some embodiments, the rhMG53 protein is
20 administered to the *ex vivo* graft for at least 1 hour.

In some embodiments, the *ex vivo* graft is an allograft or a xenograft. In some embodiments, *ex vivo* graft is a lung, liver, heart, kidney, or intestine graft.

In one aspect, disclosed herein is a method of treating a subject with primary graft dysfunction (PGD) comprising administering a recombinant human Mitsugumin-53 (rhMG53)
25 protein to the subject.

In some embodiments, the rhMG53 protein is administered in a perfusion medium.

In some embodiments, the rhMG53 comprises SEQ ID NO: 1.

In some embodiments, the rhMG53 protein is administered is administered intravenously. In some embodiments, the rhMG53 protein is administered subcutaneously.

30 In some embodiments, the rhMG53 protein is administered before transplantation of an *ex vivo* graft. In some embodiments, the rhMG53 protein is administered during transplantation of an *ex vivo* graft. In some embodiments, the rhMG53 protein is administered after transplantation of an *ex vivo* graft.

In some embodiments, the rhMG53 protein is administered to a donor before recovery. In some embodiments, the rhMG53 protein is administered to a donor during recovery.

In some embodiments, the *ex vivo* graft is an allograft or a xenograft. In some embodiments, *ex vivo* graft is a lung, liver, heart, kidney, or intestine graft.

5 In some embodiments, the subject is a human.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate several aspects described below.

10 FIGS. 1A-1B show a method schematic (FIG. 1A) and a computed tomography scan (FIG. 1B) of successful mouse lung transplant showing cuffed anastomoses.

FIGS. 2A-2D show that bronchoalveolar lavage (BAL) was collected after C57BL/6J mice underwent 1 hour of warm ischemia before lung transplantation and subsequent 5-day survival. Cell count (FIG. 2A), protein levels (FIG. 2B), LDH (FIG. 2C), and HA (FIG. 2D) were measured
15 in the BAL. Significance is denoted by $P < .05$. LDH, Lactate dehydrogenase; HA, hyaluronic acid; WT, wild type; KO, knockout; tPA, tissue plasminogen activator.

FIGS. 3A-3C show that tissue homogenates were collected after C57BL/6J mice underwent 1 hour of warm ischemia before lung transplantation and subsequent 5-day survival. FIG. 3A shows that when tissues were lysed, ET-1 expression showed no significant difference across
20 all groups. FIGS. 3B and 3C show that when assessing ET-1 and Big ET-1 levels in the BAL, WT-KO had significantly greater levels of ET-1 (FIG. 3B) and Big ET-1 (FIG. 3C) when compared with the other groups. Significance is denoted by $P < .05$. ET-1, Endothelin-1; BAL, bronchoalveolar lavage; WT-KO, wild-type knockout; tPA, tissue plasminogen activator.

FIGS. 4A-4C show representative hematoxylin and eosin-stained lung sections collected
25 after WI period, subsequent transplant, and survival indicate disrupted architecture and peri bronchial thickening in as shown in FIG. 4A. FIG. 4B shows terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining detects DNA breaks as it occurs in the last phase of apoptosis, there was increased staining in KO recipients compared with WT and tPA recipients. DAPI-stained nuclei are shown in FIG. 4C. WI, Warm ischemia; KO, knockout; WT, wild type;
30 tPA, tissue plasminogen activator.

FIGS. 5A-5B show MG53 level in BAL after WI period, subsequent transplant and survival revealed significant increase in WT-tPA compared with WT-KO as shown in FIG. 5A. FIG. 5B shows that when tissue was collected and subsequently lysed, densitometric analysis revealed a significant increase in MG53 levels in tPA recipients compared with all other groups. Significance

is denoted by $P < .05$. MG53, Mitsugumin-53; BAL, bronchoalveolar lavage; WI, warm ischemia; WT-tPA, wild type-tissue plasminogen activator; WT-KO, wild-type knockout.

FIGS. 6A-6B show representative MG53 and DAPI fluorescent staining of lung sections collected following lung transplantation in FIG. 6A. FIG. 6B shows MG53 staining and DAPI staining. Quantification of MG53 in lung tissue showed a significantly greater amount of MG53 in tPA-MG53 mice compared with the *mg53*^{-/-} mice. Significance is denoted by $P < .05$. MG53, Mitsugumin-53.

FIG. 7 shows an experimental plan detailing transplantation detail. *wt* background was used for all donors. Recipients consisted of (1) *wt*; (2) *mg53*^{-/-}; or (3) tPA-MG53. *wt*, Wild type; MG53, mitsugumin-53; tPA, tissue plasminogen activator.

FIGS. 8A-8C shows that following transplantation and survival, tissue was lysed and evaluated for pro-caspase-1 and caspase-1. FIG. 8A shows western blotting showed more pro-caspase-1 in tPA-MG53 recipients when compared with the other transplant groups. FIG. 8B shows that densitometric analysis revealed significantly greater levels of procaspase-1 in tPA-MG53 recipients as well; however, this was not true for caspase-1 as shown in FIG. 8C. tPA, Tissue plasminogen activator; MG53, mitsugumin-53; WT, wild type; KO, knockout.

FIGS. 9A-9C show that following transplantation and survival, BAL was evaluated for TNF- α and IL-1 β , whereas tissue was lysed and evaluated for pro-IL-1 β . There was no significant difference in TNF- α (FIG. 9A) or IL-1 β (FIG. 9B) levels across transplant groups. FIG. 9C shows that when tissues were lysed, and pro-IL-1 β levels were measured, there were no significant differences among groups. BAL, Bronchoalveolar lavage; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-b; WT, wild type; KO, knockout; tPA, tissue plasminogen activator.

FIG. 10 shows that following transplantation and survival, tissue was lysed and evaluated for full-length gasdermin-D and cleaved gasdermin-D. Western blotting, as confirmed by densitometric analysis, revealed no significant differences among transplant groups. GSDMD, Gasdermin-D; WT, wild type; KO, knockout; tPA, tissue plasminogen activator.

FIGS. 11A-11C show injury marker endothelin-1 (ET-1) assessment with *in vitro* porcine pulmonary artery endothelial cell (PPAEC) cultures. Untreated is vehicle treated and BSA is a protein control. FIG. 11A shows that in supernatant from PPAECs, H/R increased ET-1 release and pre-treatment with 50 μ g/mL rhMG53 or post-treatment with 10 or 50 μ g/mL rhMG53 mitigated the increase. FIG. 11B shows western blot analysis of PPAECs showed similar increases of ET-1 with H/R and decreases with rhMG53 treatment. FIG. 11C shows rapid uptake of fluorescent conjugated Alexa647-rhMG53 into the cytosol. Values in bar graphs not sharing a letter indicate a significant difference at $P < 0.05$.

FIGS. 12A-12F show assessment of therapeutic benefit of rhMG53 during rat transplantation model. FIG. 12A shows experimental overview. FIG. 12B shows wet/dry ratio is significantly changed by rhMG53 treatment ($p = 0.0089$). FIG. 12C shows Big Endothelin-1 (Big ET-1) significantly mitigated rhMG53 administration during transplantation ($p = 0.0002$). FIG. 12D shows Endothelin-1 (ET-1) significantly mitigated rhMG53 administration during transplantation ($p = 0.0194$). FIG. 12E shows representative Western blots from tissue. When cells were lysed, western blotting relative to actin loading control showed significantly reduced ET-1 levels compared to control. FIG. 12F shows densitometric quantitation showing that treatment with rhMG53 significantly reduced ET-1 levels compared to controls. Values in bar graphs (means $\pm$ SEM, $n=6$) not sharing a common letter indicate significant different at * $P<0.05$; **** $P<0.001$.

FIGS. 13A-13H show assessment of lung injury indicators in perfusate, and physiological measurements taken during and after ex vivo lung perfusion. FIG. 13A shows experimental overview. Over 120-minutes of perfusion, levels of: Big Endothelin-1 (ET-1) release during EVLP period (0-minutes $P=0.0019$, 30-minutes $P=0.028$, 60-minutes $P=0.012$, 120-minutes $P=0.0095$) shown in FIG. 13B; FIG. 13C shows ET-1 release during EVLP period (0-minutes $P=0.009$, 60-minutes $P=0.0069$, 120-minutes $P=0.0007$); FIG. 13D shows Lactate dehydrogenase (LDH) release during EVLP period (0-minutes $P=0.0019$, 30-minutes $P=0.028$, 60-minutes $P=0.012$, 120-minutes $P=0.0095$), and; FIG. 13E shows Hyaluronic acid (HA) release during EVLP period (0-minutes $P=0.0023$, 30-minutes $P=0.043$, 60-minutes $P=0.0008$, 120-minutes $P=0.0002$). All markers showed similar increases in the perfusate from 60-minutes WI lungs with rhMG53 administration mitigating the increase over the 120-minute EVLP period (LDH $p=0.047$; HA $P=0.002$; ET-1 $P=0.024$; Big ET-1 $P=0.0026$; ET-1+ Big ET-1 $P=0.0023$). Dots indicate means $\pm$ SD. At the time points indicated, * significant difference between 0h WI and 1h WI groups, # significant difference between 0h and 1h WI + rhMG53 groups, ^ significant difference between 1h WI and 1h WI + rhMG53 groups. *, #, ^ $P<0.05$; **, ##, ^^ $P<0.01$; ***, ###, ^^ = $P<0.001$; ****, ####, ^^ $P<0.001$. Physiologically, FIG. 13F shows Post-lung partial O₂ levels are significant decreased in untreated lungs and 1h WI treated with rhMG53 (0h WI $P=0.04$; 1h WI $P=0.005$); FIG. 13G shows a significant decrease in change in partial pO₂ levels was found in 1h WI group (1h WI $P=0.013$); and FIG. 13H shows decreased pulmonary vascular resistance (PVR) (0h WI $P=0.0057$; 1h WI $P<0.0001$; 1hWI + rhMG53 $P<0.0001$). Dots are individual values with means indicated by lines. Bars represent means $\pm$ SD. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

FIGS. 14A-14C show assessment of morphological and apoptotic changes after EVLP. FIG. 14A shows representative hematoxylin and eosin-stained lung sections collected after the 2h EVLP period indicate disrupted architecture and peribronchial thickening in lungs that underwent 1h of

warm ischemia (WI) prior to perfusion. In comparison, lungs that underwent 1h WI and were administered rhMG53 during the perfusion period showed more normal architecture and morphology that was similar to the perfused lungs that had experienced no WI. FIG. 14B shows Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining detects DNA breaks as it occurs in the last phase of apoptosis. TUNEL positive cells were divided by DAPI positive cells and the fold change over the mean of the 0h group was calculated (0h vs. 1h WI $P=0.0009$; 1h WI vs. 1h WI + rhMG53 $P=0.029$). FIG. 14C shows Caspase 3/7 activity was measured as an additional indicator of apoptosis, and, similar to the TUNEL staining, activity was increase in the 1h WI group and administration of rhMG53 mitigated this increase (0h vs. 1h WI $P=0.0085$; 1h WI vs. 1h WI + rhMG53 $P<0.0001$). Dots are individual values with means indicated by lines. $**P<0.001$; $***P<0.0001$; $****P<0.0001$

FIGS. 15A-15C shows assessment of MG53 levels in lung tissue and perfusate during EVLP. FIG. 15A shows Fluorescent microscopy and FIG. 15B shows quantitative analysis of fluorescent micrographs indicates an increase in MG53 in tissue of lungs that underwent 1h warm ischemia (WI) prior to perfusion with rhMG53 (1h WI vs. 1h WI + rhMG53 $P=0.009$). C) This increase in tissue MG53 is followed by a decrease of MG53 in the perfusate shows that the exogenous rhMG53 is being used by the tissue during the perfusion period (1h WI vs. 1h WI + rhMG53 at 30-minutes $P<0.0001$, 60-minutes $P<0.0001$, 120-minutes $P<0.0001$). At the time points indicated # significant difference between 0h and 1h WI + rhMG53 groups, ^ significant difference between 1h WI and 1h WI + rhMG53 groups. *, #, ^ $P<0.05$; **, ##, ^^ $P<0.01$; ***, ###, ^^= $P<0.001$; ****, ####, ^^= $P<0.001$.

FIGS. 16A-16G show assessment of therapeutic benefit of rhMG53 during porcine transplantation model. FIG. 16A shows the experimental overview. FIG. 16B shows Gross pathology of explanted lungs following reperfusion period. The left lung of the rhMG53 lung showed that the transplanted lobe had less bullae formation, erythema, and injury when treated compared to those who received vehicle. FIG. 16C shows PaO₂ in the isolated PV of the rhMG53 group (Mean (M): 445.2; Standard Deviation (SD): 55.5) compared to the vehicle group (M:201.9; SD: 182.4) at the end of the reperfusion period ($p<0.05$). FIG. 16D shows systemic PaCO₂ at the end of the reperfusion period, rhMG53 treated group (M:41.6; SD:4.6) compared to the vehicle group (M: 61.7; SD: 18.7) ($p<0.05$). FIG. 16E shows systemic pH at the end of the reperfusion period, vehicle treated group (M:7.32; SD:0.12) compared to MG53 treated group (M:7.49; SD:0.05) at the end of the reperfusion period ($p<0.05$). At the end of the experiment, there was a significantly lower release of FIG. 16F shows ET-1 in the bronchoalveolar lavage (BAL) of the rhMG53 treated pigs as compared to control. Additionally, FIG. 16G shows ET-1 release was

significantly less in the plasma as well (FIG. 17G). Significance denoted by: * < 0.05, **<0.01, ***<0.001 and ****<0.0005.

FIGS. 17A-17F show markers of injury suppressed during porcine lung transplantation by rhMG53. At the end of the experiment, there was a significantly lower release of: Tissue necrosis factor-alpha (TNF- α) as shown in FIG. 17A, receptors of advanced glycation end products (RAGE) as shown in FIG. 17B, monocyte chemoattractant factor-1 (MCP-1) as shown in FIG. 17C, plasminogen activator inhibitor 1 (PAI-1) as shown in FIG. 17D, and interleukin-18 (IL-18) in the BAL as shown in FIG. 17E. Additionally, FIG. 17F shows Lactate dehydrogenase (LDH) was assessed in plasma samples and was significantly decreased at the end of the experiment. Significance denoted by: * < 0.05, **<0.01, ***<0.001 and ****<0.0005.

FIGS. 18A-18D shows additional assessment of lung injury indicators in perfusate, and physiological measurements taken during and after ex vivo lung perfusion. FIG. 18A shows composite of ET-1 and Big-ET-1 release over EVLP period (0-minutes P<0.0001, 30-minutes P=0.0004, 60-minutes P=0.0002, 120-minutes P<0.0001). ET-1+ Big ET-1 showed increases in the perfusate from 60-minutes WI lungs with rhMG53 administration mitigating the increase over the 120-minute EVLP period (ET-1+ Big ET-1 P=0.0023). Dots indicate means $\pm$ SD. At the time points indicated, * significant difference between 0h WI and 1h WI groups, # significant difference between 0h and 1h WI + rhMG53 groups, ^ significant difference between 1h WI and 1h WI + rhMG53 groups. *, #, ^ P<0.05; **, ##, ^^ P<0.01; ***, ###, ^^= P<0.001; ****, ####, ^^ P<0.001. Physiologically, FIG. 18B shows pulmonary artery (PA) pressure at 120 minutes (0h WI P=0.002; 1h WI P=0.0031; 1hWI + rhMG53 P=0.022) and FIG. 18C shows compliance at 120 minutes increased in all groups (0h WI P<0.0001; 1h WI P=0.0005; 1hWI + rhMG53 P=0.0005). FIG. 18D shows wet/dry ratio is not significantly changed by warm ischemia (WI), rhMG53 treatment, or perfusion. Dots are individual values with means indicated by lines. Bars represent means $\pm$ SD. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

FIG. 19 shows MG53 and CD31 co-localized to damaged endothelium after injury.

FIGS. 20A-20C shows *mg53*^{-/-} with ischemic injury for 1 hour and subsequent survival for 24 hours. LDH release in the BAL and plasmas as shown in FIG. 20A; ET-1 release in the BAL and plasma as shown in FIG. 20B; HA release in the BAL and plasma as shown in FIG. 20C. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, and the middle line represents the median. The upper and lower whiskers represent maximum and minimum values, and each dot represents an individual animal. Significance denoted by *P<.05, **P<.005, ***P<.0005, ****P<.0001. LDH, Lactate dehydrogenase; BAL, bronchoalveolar lavage; ET-1,

endothelin-1; HA, hyaluronic acid; ns, nonsignificant; OD, optical density; MG53, mitsugumin-53.

FIGS. 21A-21E shows manual cell differential of BAL samples (n ¼ 5) fixed onto slides after ischemic injury for 1 hour and subsequent survival for 24 hours. FIG. 21A shows total cell count, FIG. 21B shows neutrophils per 1 mL, and FIG. 21C shows macrophages per 1 mL were lower in the exogenous MG53 treatment group but was not significant. Within the exogenous MG53 treatment group, neutrophil infiltration was significantly lower compared with macrophages per 1 mL as shown in FIG. 21D; however, this relationship did not hold true for the saline-treated group FIG. 21E. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, and the middle line represents the median. The upper and lower whiskers represent maximum and minimum values, and each dot represents an individual animal. Significance denoted by *P <.05, **P <.005, ***P <.0005, ****P <.0001. ns, Nonsignificant; MG53, mitsugumin-53.

FIGS. 22A-22B show TUNEL positive and DAPI staining in nucleus as shown in FIG. 22A. TUNEL staining detects DNA breaks as it occurs in the last phase of apoptosis. There was increased staining in the saline treatment group (left) compared with the exogenous MG53 treatment group (right). The bar graphs represent quantitative results obtained from fluorescent imaging, which show significantly higher amounts of apoptosis in the saline-treated group as shown in FIG. 22B. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, and the middle line represents the median. The upper and lower whiskers represent maximum and minimum values, and each dot represents an individual animal. Significance denoted by *P <.05, **P <.005, ***P <.0005, ****P <.0001. IRI, Ischemia-reperfusion injury; MG53, mitsugumin-53.

FIGS. 23A-23D show *mg53*^{-/-} with ischemic injury for 1 hour and subsequent survival for 24 hours. Readouts of cellular response to experimental conditions: quantitation of IL-1b levels in the plasma as shown in FIG. 23A and BAL as shown in FIG. 23B; quantitation of TNF-a levels in the plasma as shown in FIG. 23C and BAL as shown in FIG. 23D. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, and the middle line represents the median. The upper and lower whiskers represent maximum and minimum values, and each dot represents an individual animal. Significance denoted by *P <.05, **P <.01, ***P <.001, and ****P <.0005. IL, Interleukin; MG53, mitsugumin-53; TNF, tumor necrosis factor.

FIGS. 24A-24D show *mg53*^{-/-} with ischemic injury for 1 hour and subsequent survival for 24 hours. Tissue was lysed and evaluated for full-length and cleaved GSDMD: Western blotting in FIG. 24A showed more expression of full-length and cleaved GSDMD within the saline-treated group; densitometric analysis revealed significantly more full-length GSDMD as shown in FIG.

24B and cleaved GSDMD as shown in FIG. 24C expression within the saline-treated group compared with the MG53-treated group. Additionally, FIG. 24D shows tissue lysate was analyzed for ATP expression, which was significantly higher in the MG53-treated group. The upper and lower edges of the box represent the 75th and 25th percentiles, respectively, and the middle line represents the median. The upper and lower whiskers represent maximum and minimum values, and each dot represents an individual animal. Significance denoted by * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0005$. MG53, Mitsugumin-53; IRI, ischemia–reperfusion injury; GSDMD, gasdermin-D; ATP, adenosine triphosphate.

FIGS. 25A-25B show representative MG53 and CD31 fluorescent staining of lung sections collected after 1-hour ischemic injury and survival for 24 hours as shown in FIG. 25A. MG53 staining, CD31 staining, and DAPI staining are shown. The merge image of the rhMG53-treated group shows co-localization of MG53 to damaged endothelium after injury. Representative hematoxylin–eosin-stained lung sections collected after ischemia for 1 hour and subsequent survival for 24 hours in the saline and rhMG53 treatment groups, as well as quantification of MG53 in lung sections as shown in FIG. 25B. Analysis of the saline group indicates disrupted architecture and peribronchial thickening in lungs, whereas in the rhMG53 group analysis showed preserved architecture and decrease cellular infiltration. Quantification of MG53 in lung tissue showed a significantly higher amount of MG53 in the treated lung tissue compared with vehicle. Significance denoted by * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0005$. MG53, Mitsugumin-53; IRI, ischemia–reperfusion injury.

FIG. 26 shows CLAD/Bronchiolitis obliterans syndrome (BOS)-free patient survival rates of 90-day survivors, grouped by the Grade 3 PGD or not after post-bilateral lung transplantation (BLT).

FIG. 27 shows clinical manifestations of allograft dysfunction. This study aims to prevent allograft PGD through preventing vascular endothelial dysfunction, inflammasome progression and pyroptotic injury, and subsequent allograft dysfunction.

FIG. 28 shows the study overview and conceptualization of novel and innovative strategy to exploit 1) MG53 cell membrane repair and 2) MG53 modulation of IRI driven inflammation to mitigate PGD inflammatory response to produce a platform for preservation and rescue of lung allograft integrity during lung transplantation.

FIGS. 29A-29E show ET-1 elevates in EVLP perfusate (200mL vol.) after 1hr of warm ischemic injury which is mitigated with addition of 5 $\mu\text{g/mL}$ rhMG53 as shown in FIG. 29A. FIG. 29B shows IHC staining that shows ischemia induces ET-1 levels which are reduced with rhMG53 administration. FIG. 29C shows in the same lung, EVLP delivers rhMG53 which is taken up by

the lung. FIG. 29D shows in primary AoEC culture, H/R induces ET-1 elevation that is suppressed with rhMG53. FIG. 29E shows ET-1 levels in the cell culture media are also suppressed with rhMG53 treatment.

FIG. 30 shows hypoxia (3hr) and reoxygenation(3hr) causes injury to AoECs as indicated by staining with Annexin V-FITC. Alexa-647 labeling shows culture media supplemented with rhMG53 entering the cells where BSA does not.

FIGS. 31A-31C show that CRISPR knocks down VEGFR2 as shown in FIG. 31A. FIG. 31B shows a cell transfected with CRISPR-VEGFR2 does not take up rhMG53, but untransfected cell show uptake of rhMG53. Cells transfected with control CRISP (right panel) display uptake of rhMG53. FIG. 31C shows intracellular Alexa 647-rhMG53 was quantified, treatments of chloroquine or CRISPR-VEGFR2 significantly inhibited uptake of rhMG53 by MSCs (n=60 cells per group).

FIGS. 32A-32C show EaHy926 endothelial cells and THP-1 macrophages (black box) and EaHy926 with THP-1 with *MG53*^{-/-} (grey box) co-culture with H/R demonstrating their interaction resulting in increased expression of IL-1 β as shown in FIG. 32A, TNF- α as shown in FIG. 32B, and lactate dehydrogenase (LDH) as shown in FIG. 32C that is significantly reduced with exogenous rhMG53 treatment.

FIGS. 33A-33D shows activation of pyroptosis after IRI. (n=3 mice/time point). ns: not significant. **: p<0.01.

FIGS. 34A-34B show IF staining in mouse lung slides. FIGS. 34A and 34B show staining of GsdmD-N, MPO, and CD68 in mouse lung slides.

FIG. 35 shows murine lung transplantation experiment. WT lungs were transplanted to either WT (WT-WT) or *mg53*^{-/-} (WT-KO) recipients. One day after transplantation, lungs were obtained for WB analysis of GsdmD expression. n= 6 mice/group. D: donor lung, R: recipient.

FIGS. 36A-36B show IF staining and western blot images wherein LPS+Ni treatment leads to translocation of RFP-MG53 to plasma membrane as shown in FIG. 36A. FIG. 36B shows rhMG53 treatment does not change activation of caspase-1 in THP-1 cells.

FIGS. 37A-37B show western blotting images. FIG. 37A shows MG53 co-IP with GsdmD. FIG. 37B shows the effect of MG53 on caspase 1 mediated GsdmD and Sumo-GsdmD cleavage. Sumo-GsdmD a 6xHis tag and a sumo domain joined to the N-terminus of GsdmD.

FIGS. 38A-38B show the measurement of pyroptosis activity. FIG. 38A shows a schematic representation of the fluorescence liposome leakage assay. FIG. 38B shows a dose response curve of MG53 as represented by rate of leakage (pyroptosis activity).

FIGS. 39A-39D show that a 5-day survival studies were conducted with the wild-type donor to recipients of background with knockout, wild type, or over-expressor, n=4-6 as shown in FIG. 39A. At 5-days post-transplant, FIG. 39B shows the *mg53ko* recipient pairings demonstrated much higher levels of cleaved GSDM-D and FIG. 39C shows lower levels of pro-caspase 1. FIG. 39D shows that under histology, there was a reduction in TUNEL staining in the tPA recipients and increase in *mg53ko*.

FIG. 40 shows scRNA-seq, new lung atlas nomenclature (ref 63), demonstrating neutrophil enriched populations are the main cell type undergoing pyroptosis (upper right panel). The apoptotic pathway demonstrated minimal change and observed was in pneumocytes, not neutrophils.

FIGS. 41A-41F show that the etiology and mechanism of this inflammatory protection and the impact of neutrophil pyroptosis is the basis of these investigations. Murine lung transplant as shown in FIGS. 41A-41B, and survival as shown in FIG. 41C. WT donor mice lung transplantation (n=6/group) into WT (WT-WT) and *mg53*^{-/-} (WT-KO) recipient mice was conducted. FIGS. 41D-41F show that the *mg53*^{-/-} recipient demonstrates an exuberant inflammatory response.

FIGS. 42A-42C show rhMG53 delivered via EVLP to a porcine warm ischemic injury model preserves structure as shown in FIG. 42A and decreases circulating IL-6 as shown in FIG. 42B and LDH levels as shown in FIG. 42C.

FIG. 43 shows that porcine left single lung transplantation with 24 hours of cold ischemia with vehicle control and 1mg/kg rhMG53 demonstrating superior oxygenation (PaO₂) and ventilation (PaCO₂) 4 hours after reperfusion (n=5/grp)

FIGS. 44A-44D show images and graphs wherein FIG. 44A shows EVLP of human donor lungs and FIG. 44B shows TUNEL staining of normal human lung and representative samples of those unable to be rescued with EVLP demonstrating higher degree of apoptosis as shown in FIG. 44C and Caspase 3/7 activity as shown in FIG. 44D in the non-rescuable lungs over the perfusion duration of 4 hours in human EVLP validating the selected small animal biomarkers.

FIG. 45 shows serum lactate dehydrogenase levels in pigs at baseline and through the transplant and 4-hour repression period where saline vehicle control or rhMG53 was administered through central venous catheter.

FIGS. 46A-46B show tissue level analysis of explanted pig lung of the non-transplanted donor right lung (ischemic injury only control) and the transplanted left lung in the recipient (injured, reperfusion lung post-transplant) and the recipient animal native right lung (which was not transplanted or ischemic) where saline vehicle control or rhMG53 were administered through

central venous catheter. Adenosine triphosphate (ATP) (FIG. 46A) and tissue necrosis factor alpha (TNF-a) (FIG. 46B) are measured.

FIGS. 47A-47B show plasminogen activator inhibitor-1 (PAI-1) (FIG. 47A) measured in bronchioalveolar lavage fluid (BAL) (FIG. 47B) and in tissue for pig lung of the non-transplanted donor right lung (ischemic injury only control) and the transplanted left lung in the recipient (injured, reperfusion lung post-transplant) and the recipient animal native right lung (which was not transplanted or ischemic) where saline vehicle control or rhMG53 were administered through central venous catheter.

FIGS. 48A-48C show endothelin-1 (ET-1) levels in bronchioalveolar lavage fluid (BAL) (FIG. 48A), tissue (FIG. 48B), and plasma (FIG. 48C) for pig lung of the non-transplanted donor right lung (ischemic injury only control) and the transplanted left lung in the recipient (injured, reperfusion lung post-transplant) and the recipient animal native right lung (which was not transplanted or ischemic) where saline vehicle control or rhMG53 were administered through central venous catheter.

FIGS. 49A-49B show MCP-1 levels in BAL as shown in FIG. 49A and lung tissues as seen in FIG. 49B, were measured in vehicle and MG53 treated groups.

FIG. 50 shows AGER levels in BAL were measured in vehicle and MG53 treated groups.

FIGS. 51A-51B shows IL-18 levels in BAL as shown in FIG. 51A and lung tissues as shown as FIG. 51B, were measured in vehicle and MG53 treated groups.

FIGS. 52A-52B show CXCL2 levels in BAL as shown in FIG. 52A and lung tissues as shown in FIG. 52B, were measured in vehicle and MG53 treated groups.

FIGS. 53A-53B show H&E staining of lung tissues from indicated groups P1, P3.1, P3.2 and P3.3 as shown in FIG. 53A and groups P2, P4.1, P4.2 and P4.3 as shown in FIG. 53B.

FIGS. 54A-54B show H&E staining of lung tissues from indicated groups P5.1, P6.1, P6.2, P6.3, and P6.4 as shown in FIG. 54A and groups P7.1, P9.1, P9.2, P9.3, and P9.4 as shown in FIG. 54B.

FIGS. 55A-55B show H&E staining of lung tissues from indicated groups P13.1, P15.1, P15.2, P15.3, and P15.4 as shown in FIG. 55A and groups P8.1, P10.1, P10.2, P10.3, and P10.4 as shown in FIG. 55B.

FIGS. 56A-56B show H&E staining of lung tissues from indicated groups P17.1, P18.1, P18.2, P18.3, and P18.4 as shown in FIG. 56A and groups P11.1, P12.1, P12.2, P12.3, and P12.4 as shown in FIG. 56B.

FIGS. 57A-57B show H&E staining of lung tissues from indicated groups P20.1, P22.1, P22.2, P22.3, and P22.4 as shown in FIG. 57A and groups P19.1, P21.1, P21.2, P21.3, and P21.4 as shown in FIG. 57B.

FIGS. 58A-58D show PV PaO₂ levels in transplanted lung as shown in FIG. 58A, systemic arterial PaCO₂ levels as shown in FIG. 58B, and systemic arterial pH levels as shown in FIG. 58C, and LDH release in plasma as shown in FIG. 58D.

FIG. 59 shows experimental plan/model for rhMG53 administration to before/during transplant and *ex vivo* organ perfusion to determine the optimum administration time/duration.

10 DETAILED DESCRIPTION

Reference will now be made in detail to the embodiments of the invention, examples of which are illustrated in the drawings and the examples. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. The term “comprising”, and variations thereof as used herein is used synonymously with the term “including” and variations thereof and are open, non-limiting terms. Although the terms “comprising” and “including” have been used herein to describe various embodiments, the terms “consisting essentially of” and “consisting of” can be used in place of “comprising” and “including” to provide for more specific embodiments and are also disclosed. As used in this disclosure and in the appended claims, the singular forms “a,” “an,” “the”, include plural referents unless the context clearly dictates otherwise.

The following definitions are provided for the full understanding of terms used in this specification.

25 Terminology

The term “about” as used herein when referring to a measurable value such as an amount, a percentage, and the like, is meant to encompass variations of $\pm 20\%$, $\pm 10\%$, $\pm 5\%$, or $\pm 1\%$ from the measurable value.

As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a pharmaceutical carrier” includes mixtures of two or more such carriers, and the like.

Ranges can be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that when a value is disclosed that “less than or equal to” the value, “greater than or equal to the value” and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value “10” is disclosed the “less than or equal to 10” as well as “greater than or equal to 10” is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point “10” and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between two particular units is also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

“Administration” to a subject includes any route of introducing or delivering to a subject an agent. Administration can be carried out by any suitable route, including oral, topical, intravenous, subcutaneous, transcutaneous, transdermal, intramuscular, intra-joint, parenteral, intra-arteriole, intradermal, intraventricular, intracranial, intraperitoneal, intralesional, intranasal, rectal, vaginal, by inhalation, via an implanted reservoir, or via a transdermal patch, and the like.

Administration includes self-administration and the administration by another.

As used here, the terms “beneficial agent” and “active agent” are used interchangeably herein to refer to a chemical compound or composition that has a beneficial biological effect. Beneficial biological effects include both therapeutic effects, i.e., treatment of a disorder or other undesirable physiological condition, and prophylactic effects, i.e., prevention of a disorder or other undesirable physiological condition. The terms also encompass pharmaceutically acceptable, pharmacologically active derivatives of beneficial agents specifically mentioned herein, including, but not limited to, salts, esters, amides, prodrugs, active metabolites, isomers, fragments, analogs, and the like. When the terms “beneficial agent” or “active agent” are used, then, or when a particular agent is specifically identified, it is to be understood that the term includes the agent per

se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, conjugates, active metabolites, isomers, fragments, analogs, etc.

As used herein, the term “buffer” refers to a solution consisting of a mixture of acid and its conjugate base, or vice versa. The solution is used as a means of keeping the pH at a nearly constant range to be used in a wide variety of chemical and biological applications.

The phrases "concurrent administration", "administration in combination", "simultaneous administration" or "administered simultaneously" as used herein, means that the compounds are administered at the same point in time or immediately following one another.

"Comprising" is intended to mean that the compositions, methods, etc. include the recited elements, but do not exclude others. "Consisting essentially of" when used to define compositions and methods, shall mean including the recited elements, but excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions provided and/or claimed in this disclosure. Embodiments defined by each of these transition terms are within the scope of this disclosure.

A “control” is an alternative subject or sample used in an experiment for comparison purposes. A control can be "positive" or "negative."

“Composition” refers to any agent that has a beneficial biological effect. Beneficial biological effects include both therapeutic effects, e.g., treatment of a disorder or other undesirable physiological condition, and prophylactic effects, e.g., prevention of a disorder or other undesirable physiological condition (e.g., Primary Graft Dysfunction). The terms also encompass pharmaceutically acceptable, pharmacologically active derivatives of beneficial agents specifically mentioned herein, including, but not limited to, a vector, polynucleotide, cells, salts, esters, amides, proagents, active metabolites, isomers, fragments, analogs, and the like. When the term “composition” is used, then, or when a particular composition is specifically identified, it is to be understood that the term includes the composition per se as well as pharmaceutically acceptable, pharmacologically active vector, polynucleotide, salts, esters, amides, proagents, conjugates, active metabolites, isomers, fragments, analogs, etc. In some aspects, the composition disclosed herein comprises rhMG53.

The term “detect” or “detecting” refers to an output signal released for the purpose of sensing of physical phenomenon. An event or change in environment is sensed and signal output released in the form of light.

As used herein, “diagnose”, “diagnosed”, “diagnosing”, and any grammatical variations thereof as used herein, refers to the act of process of identifying the nature of an illness, disease, disorder, or condition in a subject by examination or monitoring of symptoms.

“Effective amount” of an agent refers to a sufficient amount of an agent to provide a desired effect. The amount of agent that is “effective” will vary from subject to subject, depending on many factors such as the age and general condition of the subject, the particular agent or agents, and the like. Thus, it is not always possible to specify a quantified “effective amount.” However, an appropriate “effective amount” in any subject case may be determined by one of ordinary skill in the art using routine experimentation. Also, as used herein, and unless specifically stated otherwise, an “effective amount” of an agent can also refer to an amount covering both therapeutically effective amounts and prophylactically effective amounts. An “effective amount” of an agent necessary to achieve a therapeutic effect may vary according to factors such as the age, sex, and weight of the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

As used herein, “enhance”, “enhanced”, “enhancement”, “enhancing”, and any grammatical variations thereof as used herein, refers to an act of intensifying, increasing, or further improving the quality, value, or extent of a biological function, composition, compound, cell, or tissue.

“Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA occurs.

The terms “identical” or percent “identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher identity over a specified region when

compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (see, e.g., NCBI web site or the like). Such sequences are then said to be “substantially identical.” This definition also refers to, or may be applied to, the complement of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 10 amino acids or 20 nucleotides in length, or more preferably over a region that is 10-50 amino acids or 20-50 nucleotides in length. As used herein, percent (%) amino acid sequence identity is defined as the percentage of amino acids in a candidate sequence that are identical to the amino acids in a reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

For sequence comparisons, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nuc. Acids Res.* 25:3389-3402, and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al. (1990) *J. Mol. Biol.* 215:403-410). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended

in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score.

5 Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word
10 length (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

15 The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a
20 reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01.

“Ischemia-reperfusion” refers to an occurrence when cellular dysfunction and death follow restoration of blood flow to a tissue or organ that previously experienced ischemia.

As used herein, “ischemia” refers to an inadequate blood supply to an organ or part of the
25 body. Ischemia can occur to any tissue or organ that requires or has an established blood supply, including but not limited to the heart, liver, kidneys, brain, and muscles.

The term “increased” or “increase” as used herein generally means an increase by a statically significant amount; for the avoidance of any doubt, “increased” means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at
30 least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold

increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level so long as the increase is statistically significant.

As used herein, the terms “may,” “optionally,” and “may optionally” are used interchangeably and are meant to include cases in which the condition occurs as well as cases in which the condition does not occur. Thus, for example, the statement that a formulation “may include an excipient” is meant to include cases in which the formulation includes an excipient as well as cases in which the formulation does not include an excipient.

As used herein, the term “preventing” a disease, a disorder, or unwanted physiological event in a subject refers to the prevention of a disease, a disorder, or unwanted physiological event or prevention of a symptom of a disease, a disorder, or unwanted physiological event

"Pharmaceutically acceptable" component can refer to a component that is not biologically or otherwise undesirable, i.e., the component may be incorporated into a pharmaceutical formulation of the invention and administered to a subject as described herein without causing significant undesirable biological effects or interacting in a deleterious manner with any of the other components of the formulation in which it is contained. When used in reference to administration to a human, the term generally implies the component has met the required standards of toxicological and manufacturing testing or that it is included on the Inactive Ingredient Guide prepared by the U.S. Food and Drug Administration.

"Pharmaceutically acceptable carrier" (sometimes referred to as a “carrier”) means a carrier or excipient that is useful in preparing a pharmaceutical or therapeutic composition that is generally safe and non-toxic and includes a carrier that is acceptable for veterinary and/or human pharmaceutical or therapeutic use. The terms "carrier" or "pharmaceutically acceptable carrier" can include, but are not limited to, phosphate buffered saline solution, water, emulsions (such as an oil/water or water/oil emulsion) and/or various types of wetting agents. As used herein, the term "carrier" encompasses, but is not limited to, any excipient, diluent, filler, salt, buffer, stabilizer, solubilizer, lipid, stabilizer, or other material well known in the art for use in pharmaceutical formulations and as described further herein.

The term “polypeptide” refers to a compound made up of a single chain of D- or L-amino acids or a mixture of D- and L-amino acids joined by peptide bonds.

The term “nucleic acid” as used herein means a polymer composed of nucleotides, e.g., deoxyribonucleotides or ribonucleotides.

The term "nucleobase" refers to the part of a nucleotide that bears the Watson/Crick base-pairing functionality. The most common naturally-occurring nucleobases, adenine (A), guanine

(G), uracil (U), cytosine (C), and thymine (T) bear the hydrogen-bonding functionality that binds one nucleic acid strand to another in a sequence specific manner.

The terms “ribonucleic acid” and “RNA” as used herein mean a polymer composed of ribonucleotides.

5 The terms “deoxyribonucleic acid” and “DNA” as used herein mean a polymer composed of deoxyribonucleotides.

The term “polynucleotide” refers to a single or double stranded polymer composed of nucleotide monomers.

10 The term “recombinant” refers to a human manipulated nucleic acid (e.g., polynucleotide) or a copy or complement of a human manipulated nucleic acid (e.g., polynucleotide), or if in reference to a protein (i.e., a “recombinant protein”), a protein encoded by a recombinant nucleic acid (e.g., polynucleotide). In embodiments, a recombinant expression cassette comprising a promoter operably linked to a second nucleic acid (e.g., polynucleotide) may include a promoter that is heterologous to the second nucleic acid (e.g. polynucleotide) as the result of human
15 manipulation (e.g., by methods described in Sambrook et al., *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)). In another example, a recombinant expression cassette may comprise nucleic acids (e.g., polynucleotides) combined in such a way that the nucleic acids (e.g., polynucleotides) are extremely unlikely to be found in
20 nature. For instance, human manipulated restriction sites or plasmid vector sequences may flank or separate the promoter from the second nucleic acid (e.g., polynucleotide). One of skill will recognize that nucleic acids (e.g., polynucleotides) can be manipulated in many ways and are not limited to the examples above.

25 The term “reduced”, “reduce”, “reduction”, or “decrease” as used herein generally means a decrease by a statistically significant amount. However, for avoidance of doubt, “reduced” means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (i.e. absent level as compared to a reference sample), or any decrease between 10-
30 100% as compared to a reference level so long as the decrease is statistically significant.

As used throughout, by a “subject” (or a “host”) is meant an individual. Thus, the “subject” can include, for example, domesticated animals, such as cats, dogs, etc., livestock (e.g., cattle, horses, pigs, sheep, goats, etc.), laboratory animals (e.g., mouse, rabbit, rat, guinea pig, etc.) mammals, non-human mammals, primates, non-human primates, rodents, birds, reptiles,

amphibians, fish, and any other animal. The subject can be a mammal such as a primate or a human. Administration of the therapeutic agents can be carried out at dosages and for periods of time effective for treatment of a subject.

“Therapeutically effective amount” or “therapeutically effective dose” of a composition refers to an amount that is effective to achieve a desired therapeutic result. Therapeutically effective amounts of a given therapeutic agent will typically vary with respect to factors such as the type and severity of the disorder or disease being treated and the age, gender, and weight of the subject. The term can also refer to an amount of a therapeutic agent, or a rate of delivery of a therapeutic agent (e.g., amount over time), effective to facilitate a desired therapeutic effect. The precise desired therapeutic effect will vary according to the condition to be treated, the tolerance of the subject, the agent and/or agent formulation to be administered (e.g., the potency of the therapeutic agent, the concentration of agent in the formulation, and the like), and a variety of other factors that are appreciated by those of ordinary skill in the art. In some instances, a desired biological or medical response is achieved following administration of multiple dosages of the composition to the subject over a period of days, weeks, or years.

“Therapeutic agent” refers to any composition that has a beneficial biological effect. Beneficial biological effects include both therapeutic effects, e.g., treatment of a disorder or other undesirable physiological condition, and prophylactic effects, e.g., prevention of a disorder or other undesirable physiological condition. The terms also encompass pharmaceutically acceptable, pharmacologically active derivatives of beneficial agents specifically mentioned herein, including, but not limited to, cells, salts, esters, amides, proagents, active metabolites, isomers, fragments, analogs, and the like. When the term “therapeutic agent” is used, or when a particular agent is specifically identified, it is to be understood that the term includes the agent per se as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, proagents, conjugates, active metabolites, isomers, fragments, analogs, etc.

As used herein, the terms “treating” or “treatment” of a subject includes the administration of a drug to a subject with the purpose of curing, healing, alleviating, relieving, altering, remedying, ameliorating, improving, stabilizing or affecting a disease or disorder, or a symptom of a disease or disorder. The terms “treating” and “treatment” can also refer to reduction in severity and/or frequency of symptoms, elimination of symptoms and/or underlying cause, and improvement or remediation of damage.

Disclosed herein are the components to be used to prepare the disclosed compositions as to be used in the methods disclosed herein. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are

disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. If a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if
5 each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions.
10 Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

Recombinant Human Mitsugumin-53 Compositions

15 The present disclosure discloses a composition comprising recombinant human Mitsugumin-53 (rhMG53) protein and its use thereof in treating ischemia reperfusion injury (IRI) and/or primary graft dysfunction (PGD).

Accordingly, in one aspect, disclosed herein is a perfusate composition comprising recombinant human Mitsugumin-53 (rhMG53) protein and a perfusion medium. In some
20 embodiments, the rhMG53 comprises SEQ ID NO: 1, or a fragment of variant thereof.

As used herein, Mitsugumin-53 (MG53) is a protein that functions as an essential component of plasma membrane repair. As used herein, MG53 belongs to the tripartite motif-containing (TRIM) protein family and is primarily found in skeletal muscle and alveolar epithelial cells. In some embodiments, when cell membranes are damaged, MG53 is released into the
25 bloodstream, it detects the oxidized external environment and attaches to phosphatidylserine on membrane vesicles to facilitate membrane repair. MG53 then guides these vesicles to mend the compromised membrane using a "plug and patch" approach. As disclosed herein, MG53 has been shown to have therapeutic benefit in decreased ischemia-reperfusion associated injury in both hilar clamp and transplantation models.

30 As used herein, a perfusion medium is any fluid, such as blood or saline, perfused through the blood vessels of an organ, ensuring that it receives adequate oxygen and nutrients for its proper functioning. In some embodiments, the perfusion medium is circulated through the organ using a perfusion circuit. In some embodiments, the perfusion medium is blood. In some embodiments, the perfusion medium is saline, in some embodiments, the perfusion medium is a cryo-preserved,

In some embodiments, the perfusion medium is rich in cell nutrients (such as, for example, vitamins, minerals, fatty acids and/or amino acids (such as, for example, L. Glutamine)).

As disclosed herein, the perfusate composition comprises rhMG53 and a perfusion medium, wherein the rhMG53 comprises SEQ ID NO: 1, wherein SEQ ID NO: 1 comprises a nucleotide sequence. In some embodiments, rhMG53 comprises SEQ ID NO: 2, wherein SEQ ID NO: 2 comprises an amino acid sequence. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to SEQ ID NO: 1. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to SEQ ID NO: 2.

In one aspect, disclosed herein is a preservation solution comprising recombinant human Mitsugumin-53 (rhMG53) protein and a preservation medium. In some embodiments, the rhMG53 comprises SEQ ID NO: 1.

As used herein, a preservation medium is a fluid that prevents injury to organs/grafts for transplantation during transport of the organ/graft from a donor to a recipient. In some embodiments the preservation medium is a fluid, such as, for example, the UW solution, histidine-tryptophan-ketoglutarate (HTK), and Celsior. In some embodiments, the preservation medium is a potassium-containing and hyperosmolar solution. In some embodiments, the preservation solution comprises lactobionate, raffinose, and hydroxyethyl starch as osmotic agents and other components including glutathione, adenosine, and the free-radical scavenger allopurinol. In some embodiments, the glutathione content serves to facilitate the regeneration of cellular adenosine triphosphate (ATP) and maintain membrane integrity and adenosine provides the substrate for ATP regeneration during reperfusion. In some embodiments, the preservation medium comprises tryptophan and/or ketoglutarate, wherein tryptophan serves as a membrane stabilizer and antioxidant, whereas ketoglutarate acts as a substrate for anaerobic metabolism during preservation. In some embodiments, the preservation solution comprises relatively low-potassium and/or a strong histidine buffer.

In some embodiments, the rhMG53 is administered as a recombinant protein. In some embodiments, the rhMG53 is administered through the delivery of a nucleic acid. In some embodiments, the rhMG53 is administered through the delivery of an mRNA encoding rhMG53. In some embodiments, the mRNA can include modified nucleotides. In some embodiments, the rhMG53 is administered through the delivery of a DNA sequence encoding rhMG53. In some embodiments, the rhMG53 is administered through the delivery of a nanoparticle comprising rhMG53.

As disclosed herein, the preservation solution comprises rhMG53 and a preservation medium, wherein the rhMG53 comprises SEQ ID NO: 1, wherein SEQ ID NO: 1 comprises a nucleotide sequence. In some embodiments, rhMG53 comprises SEQ ID NO: 2, wherein SEQ ID NO: 2 comprises an amino acid sequence. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to SEQ ID NO: 1. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to SEQ ID NO: 2.

In some embodiments, the perfusate composition or the preservative solution can comprise a vector (such as, for example, a plasmid, a cosmid, or an artificial chromosome) encoding rhMG53.

In some embodiments, the vector encoding rhMG53 is a viral vector.

Viral vectors are molecular tools used to deliver genetic material into cells that can be performed inside a living organism (*in vivo*), or in cell culture (*in vitro*). In some embodiments, the viral vector includes, but is not limited to adeno-associated viral (AAV) vectors, adenoviral vectors, retroviral vectors, lentiviral vectors, and hybrid viral vectors.

Method of treating or preventing ischemia reperfusion injury (IRI)

In one aspect, disclosed herein is a method of treating or preventing ischemia reperfusion injury (IRI) in an *ex vivo* graft comprising administering a recombinant human Mitsugumin-53 (rhMG53) protein to the *ex vivo* graft.

In some embodiments, the rhMG53 protein is administered in a perfusion medium of any of the preceding aspects. In some embodiments, the rhMG53 protein is administered in a preservation medium of any of the preceding aspects.

As disclosed herein, the preservation solution comprises rhMG53 and a preservation medium, wherein the rhMG53 comprises SEQ ID NO: 1, wherein SEQ ID NO: 1 comprises a nucleotide sequence. In some embodiments, rhMG53 comprises SEQ ID NO: 2, wherein SEQ ID NO: 2 comprises an amino acid sequence. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to SEQ ID NO: 1. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to SEQ ID NO: 2.

In some embodiments, the rhMG53 protein is administered to the *ex vivo* graft before reperfusion of the *ex vivo* graft. In some embodiments, the rhMG53 protein is administered to the *ex vivo* graft during reperfusion of the *ex vivo* graft.

As used herein, reperfusion is the restoration of blood flow to an organ or tissue after having been blocked due to explantation or reperfusion injury.

In some embodiments, the rhMG53 protein is administered to the *ex vivo* graft for at least 1 hour. In some embodiments, the rhMG53 is administered to the *ex vivo* graft for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 minutes or more. In some embodiments, the rhMG53 is administered to the *ex vivo* graft for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 hours or more.

As used herein, a graft is an organ or piece of tissue taken from a donor and transplanted into a recipient. In some embodiments, a graft can be an allograft or a xenograft. As used herein, the allograft is a graft taken from one subject and transplanted in a non-identical subject of the same species. As used herein, a xenograft is a graft taken from one subject and transplanted to another subject belonging to another species, e.g., animal to human.

As disclosed herein, in some embodiments, the *ex vivo* graft is an allograft or a xenograft. In some embodiments, *ex vivo* graft is a lung, liver, heart, kidney, or intestine graft. In some embodiments, *ex vivo* graft is a lung. In some embodiments, *ex vivo* graft is a liver. In some embodiments, *ex vivo* graft is a heart. In some embodiments, *ex vivo* graft is a kidney. In some
5 embodiments, *ex vivo* graft is an intestine graft.

In some embodiments, the method suppresses the ischemic reperfusion injury in an *ex vivo* graft relative to an untreated *ex vivo* graft. In some embodiments, the method suppresses the ischemic reperfusion injury in an *ex vivo* graft by 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or more relative to an untreated *ex vivo* graft.

10 **Method of treating primary graft dysfunction (PGD)**

In one aspect, disclosed herein is a method of treating a subject with primary graft dysfunction (PGD) comprising administering a recombinant human Mitsugumin-53 (rhMG53) protein to the subject.

15 In some embodiments, the rhMG53 protein is administered in a perfusion medium of any of the preceding aspects. In some embodiments, the rhMG53 protein is administered in a preservation medium of any of the preceding aspects.

As disclosed herein, the preservation solution comprises rhMG53 and a preservation medium, the rhMG53 comprises SEQ ID NO: 1, wherein SEQ ID NO: 1 comprises a nucleotide sequence. In some embodiments, rhMG53 comprises SEQ ID NO: 2, wherein SEQ ID NO: 2
20 comprises an amino acid sequence. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to
25 SEQ ID NO: 1. In some embodiments, rhMG53 comprises sequences or subsequences that are the same or have a specified percentage of amino acid residues that are the same (i.e., about 60% identity, preferably 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or of higher identity) compared to SEQ ID NO:
30 2.

In some embodiments, the rhMG53 protein is administered to the subject before transplantation of the *ex vivo* graft. In some embodiments, the rhMG53 protein is administered to

the subject during transplantation of the *ex vivo* graft. In some embodiments, the rhMG53 protein is administered to the subject after transplantation of the *ex vivo* graft.

In some embodiments, the rhMG53 protein is administered to a donor before recovery. In some embodiments, the rhMG53 protein is administered to a donor during recovery.

5 In some embodiments, the rhMG53 protein is administered to the subject for at least 1 hour. In some embodiments, the rhMG53 is administered to the subject for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 minutes or more. In some embodiments, the rhMG53 is administered to the *ex vivo* graft for 2, 3, 4, 5, 6, 7, 8,
10 9, 10, 11, 12 hours or more.

As disclosed herein, in some embodiments, the *ex vivo* graft is an allograft or a xenograft. In some embodiments, *ex vivo* graft is a lung, liver, heart, kidney, or intestine graft.

In some embodiments, the composition of any of the preceding aspects is administered in combination with an inhibitor selected from a group comprising, a white blood cell inhibitor, a
15 protease inhibitor, such as, for example, CD38 inhibitor, reactive oxygen species inhibitor, vasodilators (i.e., nitric oxide) or antioxidants, such as, for example, catalase, superoxide dismutase,

In some embodiments, the method suppresses an immune response in the subject relative to an untreated subject with graft transplantation. In some embodiments, the method suppresses the immune response in the subject by 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%,
20 55%, 60%, 65%, 70%, 75%, 80%, or more relative to an untreated subject with a graft transplantation.

In one aspect, disclosed herein is a method of improving organ/graft transplant outcomes comprising administering a recombinant human Mitsugumin-53 (rhMG53) protein. In some
25 embodiments, the organ/graft transplant is a lung, a liver, a heart, a kidney, or an intestine graft. In some embodiments, the organ/graft transplant is a lung.

EXAMPLES

The following examples are set forth below to illustrate the compositions, cells, methods, and results according to the disclosed subject matter. These examples are not intended to be
30 inclusive of all aspects of the subject matter disclosed herein, but rather to illustrate representative methods and results. These examples are not intended to exclude equivalents and variations of the present invention which are apparent to one skilled in the art.

Example 1: MG53 mitigates warm ischemic lung injury in a murine model of transplantation.

Lung allografts are often rejected due to the fear of adverse postoperative outcomes. The lack of comprehension regarding the molecular processes involved in lung injury and repair poses a hindrance to creating innovative treatments for lung transplantation. This study shows that MG53 could be used during transplantation to mitigate injury and enhance transplantation outcomes.

Lung transplantation is limited by the lack of suitable donors. Currently, only 30% of offered allografts are accepted. Allografts are often rejected due to fears of organ quality negatively affected by hypoxia, edema, or trauma. Although there are many factors that contribute to poor organ quality and thus their rejection, ischemia–reperfusion injury (IRI) is a major contributor to poor allograft function and quality. Furthermore, IRI is often identified as the underlying cause of primary graft dysfunction (PGD) in lung allografts. Unfortunately, PGD is a relatively common adverse event that occurs in up to 30% of patients and has significant effects on mortality and morbidity. Therefore, therapeutics targeting the 70% of rejected allografts that require further repair and resuscitation, expand the donor pool. Repairing the damage these marginal allografts sustain from IRI upon allograft implantation is critical to patient safety and longevity.

To accomplish this, exploiting endothelial cell membrane injury repair pathways offer a novel means of salvaging marginal donor lungs by reducing IRI-associated damage and could possibly prevent PGD after transplantation. Mitsugumin-53 (MG53) is a protein that functions as an essential component of plasma membrane repair. Previous studies have shown that MG53 can reduce ventilator-associated lung injury as well as IRI in transplant relevant models. Modulation or administration of MG53 and its related cell membrane repair pathway is potentially a novel therapeutic for the treatment of IRI-related PGD during lung transplantation. It is hypothesized that MG53-related repair of the cell membrane is an integral component of protection against IRI, and therefore when wild-type lungs are transplanted into genetically absent or overexpressing recipients of MG53, the severity of injury is most pronounced in the genetically absent recipient.

Lung transplant warm ischemia–reperfusion injury (IRI) results in cellular injury, inflammation, and poor graft function. Mitsugumin-53 (MG53) is an endogenous protein with cell membrane repair properties and the ability to modulate the inflammasome. Disclosed herein is that the absence of circulating MG53 protein in the recipient increases IRI, and higher levels of circulating MG53 protein mitigate IRI associated with lung transplantation and that MG53 functions as a therapeutic reagent to rescue marginal allografts and to prevent ischemia reperfusion injury–related primary graft dysfunction during lung transplantation.

Methods:

To demonstrate protection, wild-type (*wt*) lung donor allografts were transplanted into a *wt* background, a MG53 knockout (*mg53*^{-/-}), or a constitutively overexpressed MG53 (tissue plasminogen activator-MG53) recipient mouse after 1 hour of warm ischemic injury. Mice survived for 5 days after transplantation. Bronchioalveolar lavage, serum, and tissue were collected at sacrifice. Bronchioalveolar lavage, serum, and tissue markers of apoptosis and a biometric profile of lung health were analyzed.

Animals: All in vivo experiments used C57BL/6J mice (The Jackson Laboratory) weighing 20 to 30 g. MG53 knockout (*mg53*^{-/-}), overexpressed MG53 (tissue plasminogen activator [tPA]-MG53), and wild-type (*wt*) mice were bred and maintained as previously described. The animals were housed under standard conditions (humidity: 45%-70%, temperature: 21 ± 3 °C, 12-h light-dark cycle) and provided ad libitum access to water and standard diet. Animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee (protocol no. 2012A00000135). All experiments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 8023, revised 1978).

Mice Lung Transplantation: For transplant experiments, *wt* donor mice, all with 1 hour of warm ischemic injury, were randomly assigned to 1 of 3 recipient groups with the following backgrounds (Figure 7): (1) *wt* background (control) (n ¼ 6/group); (2) *mg53*^{-/-} background (n ¼ 4/group); (3) tPA-MG53 background (n ¼ 6/group). Protocols for a donor lung harvest and subsequent transplantation of the donor graft into the recipient have been described earlier. To summarize, mice were administered ketamine and xylazine (Henry Schein). When a surgical plane of anesthesia was met, a tracheostomy tube was placed and a thoracoabdominal incision was made. The diaphragm was then carefully dissected along the thoracic arch, and the thoracic cavity was further exposed by median sternotomy. The mice were then sacrificed by cutting the inferior vena cava (IVC). While the lungs were still ventilating, the right and left ventricles were cut, and the lungs were gravity flushed with preservation solution (Perfadex; XVIVO) with an angiocatheter that was introduced directly into the pulmonary artery (PA). The ventilator was then disconnected from the endotracheal tube inflated with a predetermined volume. A clamp was placed on the trachea to keep the lungs inflated. The heart-lung block was then removed and wrapped in a moistened sterile cotton gauze and left on a warm surgical board (37 °C) for 1 hour to induce warm ischemia in the lungs. After 1 hour of warm ischemia, the heart-lung block was placed on a sterile gauze dampened with ice cold phosphate-buffered saline (Fisher BioReagents) on a sterile

Petri dish on ice. The pulmonary ligaments were then carefully incised to separate the left lung from the esophagus and the postcaval lobe. The hilar area of the left lung was then carefully trimmed, and the left pulmonary vein (PV), PA, and bronchus (Br) were isolated. Appropriately sized angiocatheter cuffs were chosen and then placed inside the PV, PA, Br, and secured with suture.

5 The donor lung was again covered with moistened sterile gauze and kept on ice until ready for transplantation to the recipient mouse. The recipient mouse was then prepared for transplantation in a standard fashion. Once the lung of the recipient mice was removed, the donor allograft was then transplanted into the recipient by anastomosing the PV, Br, and PA, respectively, using suture to secure each anastomosis over the previously mentioned cuff. The PA and PV were flushed with
10 heparinized saline before anastomosis. The clamp around the hilum was then removed, allowing reperfusion and ventilation of the transplanted donor lung. The thoracotomy incision was then closed in 2 layers using nylon suture. The mice then survived for 5 days under standard conditions according to the Institutional Animal Care and Use Committee protocol. Successful demonstration of transplanted lobe using the modified cuff technique.

15 At sacrifice, after a 5-day survival, the mice were then prepped and anesthetized as described earlier. A midline abdominal laparotomy was performed, and 0.5 mL of blood was collected from the IVC, for further biochemical analysis. The mice were subsequently humanely killed by cutting the IVC. The diaphragm was then dissected along the thoracic arch and the thoracic cavity was exposed by median sternotomy. A bronchoalveolar lavage (BAL) was performed by filling a
20 syringe with 0.8 mL of sterile saline, injecting it into the endotracheal tube, and withdrawing twice. The right and left ventricles were cut, and the lungs were flushed by gravity through the pulmonary artery using an angiocatheter with 5 mL of prechilled phosphate-buffered saline solution (GIBCO). For the right lung of the recipient: the superior lobe and the postcaval lobe were snap frozen for protein and RNA expression analysis, the middle lobe was preserved in 10% neutral buffered
25 formalin for histology and the inferior lobe was used for wet to dry weight ratio. The left lung of the recipient was divided into 3 parts: the upper region was collected for snap freeze, the middle region was used for histology, and the lower region was used for wet-to-dry weight ratio.

Biochemical Assays: Colorimetry and fluorescence for all biochemical assays were measured using the POLARstar Omega multimode microplate reader (BMG LABTECH).
30 Enzyme-linked immunosorbent assay. Supernatants were collected from transplanted and native lungs. The levels of endothelin-1 (ET-1), Big-ET-1 (Enzo Life Sciences), hyaluronic acid (HA), tumor necrosis factor-alpha (TNF-a), and interleukin-1-beta (IL-1b) (R&D Systems) were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Lactate dehydrogenase (LDH) release assay: LDH release was measured in perfusate using the LDH cytotoxicity detection assay kit (Clontech Laboratories) according to the manufacturer's instructions. The optical density values were analyzed at 490 or 492 nm by subtracting the reference value at 620 nm.

5 **Western Blot Analysis:** Western blot analysis was performed using lung tissue samples. Lung tissue was homogenized in radioimmunoprecipitation assay lysis buffer (Millipore) with a 1:3 protease inhibitor cocktail (Cell Signaling Technology). Equal amounts of lysate proteins were electrophoresed on 6% to 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking
10 membranes in a Tris-buffered saline/0.1% Tween 20 supplemented with 5% nonfat dry milk for 3 hours. Membranes were incubated with primary mouse antibody against ET-1, gasdermin D (Abcam) IL-1b, and cleaved caspase-1 (Cell Signaling Technology). The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. Protein levels on the membrane were determined
15 using Western Lightning Plus-ECL (PerkinElmer), and autoradiography film (Denville Scientific). Incubation with monoclonal mouse b-actin antibody (Sigma-Aldrich) was conducted for comparative control. Quantitative results were obtained using Image J software (National Institutes of Health).

20 **Histologic Analysis and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining:** Histological samples were stored in 10% formalin, transferred to ethanol for paraffin embedding, and 5-mm sections were cut and processed. Hematoxylin and eosin and immunohistochemical staining was performed using standard methods. In addition, unstained slides were used for MG53 immunohistochemical quantification. Apoptotic cells in the lung tissue section were determined using the In Situ Cell Death Detection Kit (Roche) according to the
25 manufacturer's instructions, which is a TUNEL assay that identifies apoptotic cells by labeling DNA fragmentation sites.

30 **MG53 Staining:** For the immunohistochemical analysis, paraffin-embedded, formalin-fixed sections of murine lung were cut from tissue blocks at 4- to 6-mm thickness and mounted on slides. Mounted tissue sections were heated at 60°C for 30 minutes and washed 4 times in xylene each for 10 minutes to remove the paraffin wax, followed by rehydration through an ethanol series, before washing and storing in dH₂O until use. Antigen retrieval was conducted via a 30-minute incubation in 100°C sodium citrate buffer followed by preincubation in 1% fetal bovine serum/glycine blocking solution. A specific primary antibody against MG53 (1:200, rabbit,

homemade) incubated with the tissue section overnight. The details for creation of the homemade MG53 primary antibody have been described in detail before. For the visualization, tissue sections were stained with Alexa Fluor 647 (rabbit) and Alexa Fluor 555 (rat) second antibodies (1:500, A32733, Invitrogen; 1:500, A21434, Invitrogen). The stained tissue sections were mounted in DAPI Fluoromount-G (0100-20; Southern Biotech). Images were taken using the Nikon A1R-MP Laser Scanning Confocal Microscope.

Statistical Analyses: In vitro experiments were repeated in triplicate to assess consistency and reliability, and representative data sets were displayed. The results were expressed as mean $\pm$ standard deviation for each treatment group in each experiment. Significance was determined between 2 groups with a 2-tailed Student *t* test or among 3 or more groups with a one-way analysis of variance with Dunnett post hoc analysis to control for the familywise error rate associated with multiple comparisons of the experimental treatment groups to the untreated group. Analyses were performed using GraphPad Prism 7 software.

Results:

mg53^{-/-} mice had significantly greater levels of markers of overall cell lysis and endothelial cell injury. Overexpression of MG53 resulted in a signature similar to that of *wt* controls. At the time of explant, tissue plasminogen activator-MG53 recipient tissue expressed significantly greater levels of MG53, measured by immunohistochemistry, compared with *mg53*^{-/-}, demonstrating up- take of endogenous overexpressed MG53 into donor tissue.

Markers of Injury in BAL: Following transplantation, and 5-day survival, *wt* lungs that were transplanted into *mg53*^{-/-} had significantly greater cell counts in their BAL fluid compared with *wt*-*wt* and tPA-MG53 transplantation (Figure 2A). The total protein measured in BAL was significantly increased in the *wt*-*mg53*^{-/-} transplantation group compared with the other groups (Figure 2B). Furthermore, LDH in BAL was also significantly greater in the *wt*-*mg53*^{-/-} group (Figure 2C). The HA found in the BAL was not significantly different among the groups (Figure 2D).

Endothelial Damage Mitigated by Endogenous MG53: When tissue was homogenized and lysed, Western blot analysis showed that there were no significant differences in ET-1 expressed in donor or recipient lungs in any of the transplant groups (Figure 3A). When analyzing BAL fluid at the end of the survival period, recipient *wt* and tPA-MG53 mice had significantly decreased levels of ET-1 (Figure 3B) and Big ET-1 (Figure 3C) in the BAL.

Histopathologic and Apoptotic Marker Changes With Endogenous MG53:

Hematoxylin and eosin histological staining of lung sections showed that compared to *mg53*^{-/-} recipients, *wt* and tPA-MG53 recipients suffered less injury, indicated by decreased disruption of tissue architecture and peribronchial thickening (Figure 4A). The TUNEL staining of lung sections indicated that apoptosis was also increased in the *mg53*^{-/-} recipient group and decreased mitigated by endogenous MG53 in the *wt* and tPA-MG53 recipient groups (Figure 4B). As reference, nuclei are DAPI stained (Figure 4C). Additionally, pro-caspase-1, caspase-1, TNF- α , IL-1 β , gasdermin D activity, as biochemical indicators of programmed cell death, were also measured. When tissue was homogenized, pro-caspase-1 and caspase 1 levels were analyzed via Western blotting (Figure 8). Pro-caspase-1 was greater in the tPA-MG53 recipient lobe as compared to the both the *wt* donor lobe and *mg53*^{-/-} recipient lobe (Figure 8B). Caspase-1 levels were not significantly different among the groups (Figure 8C). Among the transplantation groups, there were no significant differences in TNF- α levels in the BAL or IL-1 β levels in BAL or tissue (Figure 9). Gasdermin-D and cleaved gasdermin-D levels were not significantly different amongst groups either (Figure 10).

MG53 Quantification and Expression in Recipients: The quantification of MG53 in recipient BAL revealed a significantly greater amount in tPA-MG53 recipients compared with *mg53*^{-/-} recipients (Figure 5A). In tissue homogenates of *wt* donor lung tissue, those that were transplanted into tPA-MG53 recipients had significantly increased levels of MG53 when compared with all groups (Figure 5B). Fluorescent microscopy of lung tissue sections following transplantation stained for MG53 and DAPI (4',6-diamidino-2-phenylindole), and the resulting merged image can be seen in Figure 6, A. Imaging demonstrated that *mg53*^{-/-} mice had no background MG53 expression, whereas tPA-MG53 mice displayed endogenous MG53 expression and presence in the lungs, and presumably site of injury. MG53 intensity was significantly elevated in the transplanted lobe in the *wt* to tPA-MG53 group compared to the *wt* to *mg53*^{-/-} group (Figure 6B).

Discussion:

This study shows that MG53 is an integral part of the cell membrane repair machinery and can be used for the use of new therapeutics in lung transplantation.

Previous observations have noted that MG53 is initially involved in cell membrane repair and machinery. To summarize, when cell membrane damage occurs, MG53 senses the oxidized environment outside the cell and binds to phospholipids on membrane vesicles within the cytoplasm. MG53 then brings these vesicles to the damaged membrane and facilitates their repair through a

“plug-and-patch” mechanism. Protection of the endothelium is paramount, as damage from IRI disrupts this barrier, leading to migration of immune cells into the alveolar space causing further edema, injury, and eventually can manifest clinically as PGD. After 1 hour of warm ischemia, subsequent transplantation, and a 5-day survival, circulating MG53 was still found in quantifiable levels within donor lung tissue. MG53 staining confirmed the protein was still visualized within the lung parenchyma as well and further confirmed that it was congregated around the alveoli and presumably laying within the endothelium, i.e., the site of injury. This confirms previous studies and shows a finding of prolonged presence of MG53 in donor tissues. This confirms that MG53 not only has the ability to migrate to the affected endothelial cells during injury but also can be present for a sustained period of time contributing to further repair and regeneration.

Although showing the circulation of endogenous MG53 to donor tissue is important for studies that incorporate exogenous MG53 administration, validating MG53’s ability to mitigate and modify established markers of injury is equally important. IRI is a known contributor to PGD and is mediated by inflammatory signaling cascades that lead to impaired cell function, resulting in endothelial cell death. During these cascades, markers of inflammation and cell damage are released. In this study, markers of acute lung injury, cellular integrity, and death were specifically looked at. ET-1 is a marker of endothelial cell integrity during lung injury, and previously high levels of ET-1 have been shown to correlate with the development of PGD. ET-1 has also been studied at several time points throughout transplantation: before organ procurement, intraoperatively, and immediately postoperatively. In this study, ET-1 and Big ET-1 (amino acid from which ET-1 is cleaved) were found at significantly greater levels in BAL of the MG53 knockout transplantation group compared with MG53 overexpressers and control. These results overall convey that MG53 has the ability to maintain cell integrity. LDH is a widely recognized marker of cellular inflammation and damage, and specifically in transplantation models, it has been found at substantially lower levels of perfusate in the lungs that are considered suitable for transplantation post-ex vivo lung perfusion. Post transplantation, acute rejection is often accompanied by an increase in serum LDH. Endogenous MG53 was able to mitigate cellular inflammation in both *wt* and tPA-MG53 recipients. Apoptosis is a major type of cell loss and contributes to IRI during lung transplantation. Quantification of cell death (i.e., apoptosis) was evaluated by TUNEL staining, which showed an increase in intensity in *mg53*^{-/-} recipients compared with *wt* and tPA-MG53 recipients.

In this 5-day survival model, there was no significant difference in ET-1 expression in the tissue, in addition to HA, TNF- α , caspase-1, IL- β , and gasdermin D. These markers represent acute inflammatory response as well as pyroptosis. Pyroptosis is another form of cell death that is triggered

in response to IRI, and, unlike apoptosis, arises from cell membrane disruption and mitochondria dysfunction. Previously, exogenous MG53 administration has been shown to decrease the pyroptotic effects of IRI. However, after 5 days, the resolution of acute inflammation, as well as the cessation of ongoing cellular death are observed.

Overall, endogenous circulating MG53 can be delivered effectively to the lung transplanted after warm ischemic injury and due to the endogenous nature of the recipient animal, the effect persists after injury and preserve cellular integrity, decrease cellular inflammation, and mitigate apoptosis. In this transplantation model, all recipients received *wt* lung allografts, which have the ability to produce endogenous MG53 from their endothelium. However, these cells can only produce finite quantities of MG53, which fall well below normal circulating levels in mice, thus limiting the ability to prevent injury when transplanted into *mg53*^{-/-} recipients as evident by this study. Here, it is showed that when *wt* allografts transplanted into mice that express MG53 at normal (*wt*) or higher levels (tPA), it was the endogenously produced MG53 from the recipient that was able to mitigate lung injury, as well as forms of prograded cell death.

Conclusions:

In a warm IRI model of lung transplantation, the absence of MG53 resulted in increased cell injury and inflammation. Overexpression of MG53 in the recipient results in protection in the *wt* donor. Collectively, these data demonstrate that circulating MG53 is protective against IRI-mediated injury and specifically circulating MG53 migrates to the site of lung injury and remains present for a sustained period of time. These results show that MG53 can mitigate lung injury caused by warm ischemia during lung transplantation, shows a treatment for IRI, prevention of PGD, and a novel therapeutic in the field of organ transplantation.

Example 2: MG53 preserves endothelia integrity to protect against ischemia reperfusion injury during lung transplantation .

Lung transplantation is hampered by a lack of suitable donors, which is highlighted by the fact that only around 20% of eligible donor allografts end up being successfully transplanted. In an effort to expand the donor pool, extended criteria donors are being used with increasing frequency, however, have been shown to have worse peri-operative outcomes and decreased survival. On a molecular level, these allografts suffer disproportionately worse ischemia reperfusion injury (IRI). The result of this injury is the breakdown of the endothelial barrier, while also for innate immune cells to populate the alveolar space and cause further damage to the lung parenchyma. Clinically, this results in poor allograft function and primary graft dysfunction (PGD)

following transplantation. PGD is a significant adverse event that occurs after lung transplantation, and unfortunately can affect up to 30% of patients leading to unnecessary morbidity and mortality. Thus, protecting the endothelium during transplantation is critical to preventing the sequela of IRI and thus mitigating PGD.

5 Targeting endothelial cell membrane injury repair pathways may offer an effective means to rescue the donor allograft and protect against the development of PGD after transplantation. Mitsugumin-53 (MG53), is a protein which functions as an essential component of plasma membrane repair, which has shown previously to have therapeutic benefit in decreased ischemia-reperfusion associated injury in both hilar clamp and transplantation models. MG53 belongs to the
10 tripartite motif-containing (TRIM) protein family and is primarily found in skeletal muscle and alveolar epithelial cells. When cell membranes are damaged, MG53 is released into the bloodstream to facilitate membrane repair. The mechanism by which MG53 repairs cell membranes is well documented. Briefly, when plasma membranes are disrupted, MG53 detects the oxidized external environment and attaches to phosphatidylserine on membrane vesicles. It
15 then guides these vesicles to mend the compromised membrane using a "plug and patch" approach.

Therapeutic approaches to modulate MG53 function or systemic administration of recombinant human MG53 (rhMG53) protein is potentially a safe biologic reagent for treatment of transplant related IRI, and thus prevention of PGD. It was hypothesized that MG53-mediated repair constitutes an integral component of lung protection through preservation of endothelial cell
20 integrity during allograft transplantation and can rescue marginal allografts by protecting against IRI during lung transplantation. In order to test this hypothesis, two well-established and complimentary models of IRI were employed, further validated rhMG53 administration in small animal transplantation which culminated in successful porcine lung transplantation.

Methods:

25 **Animals:** Experiments used male Sprague–Dawley rats (Envigo, Indianapolis, IN) weighing 225-250g or Yorkshire Swine (Fannin Farm, Hillsboro, OH) weighing 25-35kg. Animals were housed under standard conditions and were provided with *ad libitum* access to water and standard chow diet. Animal experiments were humanely performed with approval from the Institutional Animal Care and Use Committee (IACUC protocol #2012A00000135). All
30 experiments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Endothelial cell primary culture: Primary porcine pulmonary artery endothelial cells (PPAEC) were harvested as previously described. Briefly, fresh pulmonary artery (PA; 10-14 cm),

was sourced from heparinized and euthanized pigs, and PPAEC were isolated by suturing and clamping off the branches and ends of the vessels, and filling the PA with collagenase (Sigma, St. Louis, MO) for 15 minutes. The endothelium was then denuded with gentle scraping and cells were suspended in 20% FBS (Lonza, Walkersville, MD) before centrifuging at 200 G for 5 minutes.

5 Cells were cultured in MEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza) with bovine brain extract at 37°C in a humidified atmosphere of 5% CO₂ in air until confluent.

Hypoxia, Reoxygenation and Normoxia Model: PPAEC were plated overnight (3.5x10⁵ cells/well; 6 well plate; n=4 samples/group) at 37°C in normoxic conditions in a humidified atmosphere of 5% CO₂/95% air. For hypoxia conditions by filling an Atmospheric Control Unit (BGM LABTECH, Ortenberg, DE) with N₂ gas (hypoxia). For the pretreatment group, cells were treated with rhMG53 (10 or 50 µg/ml) and incubated in hypoxic conditions. For the post-treatment group, after 3h of hypoxia, cells were washed and treated with rhMG35 (10 or 50 µg/ml) and then incubated in normoxic conditions (reoxygenation).

15 PPAECs (3.5x10⁵ cells/well, n=4 samples/group) were seeded in 35mm glass-bottom culture dishes. After 24 hours, the culture medium was replaced with the fresh medium. The cells were then incubated overnight in a hypoxic chamber set to 1% O₂, 5% CO₂, and 94% N₂ (Biospherix hypoxia chamber). Following hypoxia exposure, the cells were treated with Alexa647-conjugated rhMG53 at a concentration of 20 µg/ml. Protein uptake was subsequently examined using a Nikon A1R confocal microscope.

Rat Ex Vivo Lung Perfusion: For EVLP experiments, rats were randomly assigned to 1 of 3 groups (n=6/group): (1) no (0h) ischemic injury prior to 2h of perfusion with control perfusate; (2) 1h of warm ischemic time prior to 2h of EVLP with control perfusate; and (3) 1h of warm ischemic time prior to 2h of EVLP with rhMG53 perfusate.

25 Detailed protocols to extract the heart-lung block and perform EVLP are described previously in detail. Briefly, when a surgical plane of anesthesia was met, a tracheostomy tube was placed, the thoracic cavity was opened, and heparin (100U/kg) was administered. The lungs were inflated with room air (3 mL/kg) and the rat was euthanized. The pulmonary artery (PA) and left atrium (LA) were cannulated, and the lungs were gravity flushed with cold preservation solution (Perfadex, XVIVO, Gothenburg, SE). Following the ischemic time, the heart-lung bloc was connected to the EVLP Isolated Lung System (Harvard Apparatus, Holliston MA). The control perfusate consisted of 4% human serum albumin (CLS Behring, King of Prussia, PA), Williams'

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Medium (Sigma), and heparin (0.01 units/mL). rhMG53 perfusate consisted of the control perfusate with 50ug/mL of rhMG53.

Ventilation parameters were then set with a flow speed initially 5-10% predicted cardiac output and increased to 20% of predicted cardiac output no later than 15 minutes after beginning perfusion. Physiologic lung parameters were measured continuously using Power Lab software (AdInstruments, Sydney, AU). The perfusate was sampled immediately before entering the PA or after exiting the LA via sample ports at 0h, 1h, and 2h and analyzed by a blood gas analyzer (ABL-90 Flex Plus, Radiometer American Inc., Brea, CA). Additionally, an aliquot was frozen for further analysis. Tissue was harvested and processed immediately following perfusion. The right upper lobe was used for wet-to-dry weight ratio. The middle right lobe was fixed in 10% neutral buffered formalin. The lower right lobe, upper left lobe, and lower left lobe were snap frozen.

Rat Lung Transplantation: For rat lung transplant experiments, Sprague–Dawley rats were randomly assigned to 1 of 2 groups (n=6/group): (1) 1h ischemic injury prior to transplantation with administration of rhMG53 (1 mg/kg in 0.1 mL/kg of normal saline); (2) 1h ischemic injury prior to transplantation with administration of saline (control, 0.1 mL/kg).

Protocols to perform a donor lung harvest, and subsequent transplantation of the donor allograft into the recipient have been described previously. Donor lung allografts were procured and preserved in the standard fashion. When explanted, the lungs were filled with room air (3 mL/kg) covered in moistened gauze and left on a warming surgery board (37°C) for 1h to induce ischemia. After 1h of warm ischemia, a left thoracotomy was then performed on the recipient rat and prepared for transplantation in the standard fashion. The donor PA and PV were flushed with 0.2 mL of heparinized saline (1 U/mL) prior to anastomosis, and allograft was then transplanted into the recipient. The clamp around the hilum was removed, allowing for reperfusion and ventilation of the donor lung. The thoracotomy and laparotomy incisions were then closed using nylon suture. The rats then survived for 3h under ventilation and anesthesia. After 3h hours of reperfusion blood was collected from the recipients for further analysis. The recipient was subsequently euthanized, and tissue was harvested and processed immediately. The recipient's left and right lungs were divided into three parts: the upper region was collected for snap-frozen, the middle region was used for histology, and the lower region was used for wet-to-dry weight ratio.

Porcine Lung Transplantation: For porcine lung transplantation experiments, Yorkshire swine were randomly assigned to 1 of 2 treatment groups (n=6/group): rhMG53 (1mg/kg) administration or vehicle control (saline). Protocols for porcine lung transplantation have been described previously.

Briefly, donors and recipients were size matched so the weight difference between the two was no greater than 10 kg. A median sternotomy was performed, heparin was administered (300U/kg) and the PA was cannulated. The pig was then euthanized via exsanguination and lungs were flushed with 2L of pre-chilled preservation solution (Perfadex, XVIVO) mixed with prostaglandin (500mg) via the PA. The lungs were then explanted en bloc and a back table retrograde flush was performed with the same preservation solution as before. The lung block was then triple-bagged and preserved on ice for 24 hours.

The following day, the recipient was anesthetized, lined up for invasive monitoring, prepped, and draped in standard sterile fashion. The donor allograft was prepared on the back table, and a BAL sample was taken from the right donor lung for a baseline injury profile. Following heparin administration, a recipient left pneumonectomy was performed. A left lung transplant was performed, and prior to completion of anastomosis, methyl-prednisone and either rhMG53 or control was administered systemically via the jugular vein. The chest was left open, and the pig survived under anesthesia for 4 hours. During this time, blood samples were obtained via central access for analysis (ABL-90 Flex PI, Radiometer American Inc., Brea, CA). Prior to sacrifice, isolated left and right PV blood gases were obtained. At sacrifice, tissue and BAL samples were taken from the right native lung and left donor allograft. To avoid compounding injury from dependent atelectasis, the lower lobes of the animal were not used for sample collection. The right and left middle lobes (or middle lobe equivalent) were used for BAL collection, while the left and right upper lobes were used for snap-frozen, histology, and wet-to-dry weight ratio.

Biochemical Assays: Colorimetry and fluorescence for all assays were measured using the POLARstar Omega multi-mode microplate reader (BMG LABTECH, Stafford, TX). ET-1 (Enzo Life Sciences, Farmingdale, NY), Big ET-1 (Enzo Life Sciences), and HA (R&D Systems, Minneapolis, MN) levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. LDH release was measured using the LDH cytotoxicity detection assay kit (Clontech Laboratories, Mountain View, CA) according to the manufacturer's instructions. The optical density values were analyzed at 490 or 492 nm by subtracting the reference value at 620 nm. Caspase-3/7 activity was detected in lung tissue extracts with the Apo-ONE Homogeneous Caspase-3/7 kit (Promega, Madison, WI) according to the manufacturer's instructions.

Western blot analysis: Western blot analysis was performed using culture media and cell lysates to evaluate ET-1 (Abcam, Cambridge, UK) and β -actin (Sigma, St. Louis, MO) as a comparative control. Equal amounts of supernatants or proteins were electrophoresed on 6–12%

SDS-PAGE gels and transferred onto a nitrocellulose membrane. Membranes were blocked in a Tris-buffered saline/0.1% Tween 20 and 5% nonfat dry milk for 3h, incubated with primary antibodies for 2h at room temperature or overnight at 4°C, and incubated with horseradish peroxidase–conjugated secondary antibodies for 1h at room temperature. Western blots were imaged using Western Lightning Plus-ECL (PerkinElmer, Waltham, MA) and autoradiography film (Denville Scientific, Metuchen, NJ), and quantified by using ImageJ (NIH, Bethesda, MD).

TUNEL staining: Apoptotic cells in lung tissue section were determined using the In Situ Cell Death Detection kit (Roche, Indianapolis, IN), which is a TUNEL assay. Tissues were also stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. The TUNEL and DAPI images were merged to allow for visual identification and quantification of apoptotic cells by ImageJ (National Institute of Health) analysis.

Histological Analysis: Histologic specimens were stored in 10% formalin, transferred to ethanol for paraffin embedding, and 5-µm sections were cut and processed. Hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining was performed using standard methods.

For the immunohistochemical analysis, paraffin-embedded formalin-fixed sections of murine lung were cut from tissue blocks at 4–6µm thickness and mounted on slides. Mounted tissue sections were heated at 60°C for 30 min and washed four times in xylene each for 10 min to remove the paraffin wax, followed by rehydration through an ethanol (EtOH) series, before washing and storing in dH₂O until use. Antigen retrieval was conducted via 30 min incubation in 100°C sodium citrate buffer followed by pre-incubation in 1% FBS/glycine blocking solution. A specific primary antibody against MG53 (1:200, Rabbit, Homemade) incubated with the tissue section overnight. The details for creation of the homemade MG53 primary antibody have been described in detail before. For the visualization, tissue sections were stained with Alexa Fluor 647 (Rabbit) and Alexa Fluor 555 (Rat) second antibodies (1:500, A32733, Invitrogen; 1:500, A21434, Invitrogen). The stained tissue sections were mounted in DAPI Fluoromount-G (0100-20, Southern Biotech). Images were taken using the Nikon A1R-MP Laser Scanning Confocal Microscope.

Statistical Analyses: In vitro experiments were repeated three times to test for consistency and reliability and representative data sets were displayed. EVLP studies had four biological replicates, and assays were run in technical triplicates. The results were expressed as mean ± SD for each treatment group in each experiment. Significance with determined between two groups with a two-tailed Student's t-test or among 3 or more groups with a one-way ANOVA with Dunnett's post hoc analysis to control for the familywise error rate associated with multiple

comparisons of the experimental treatment groups to the untreated group. P-values less than 0.05 were statistically significant. Analyses were performed using GraphPad Prism 7® software (LaJolla, CA).

Results:

rhMG53 Modulates Injury Marker Endothelin-1 In Primary Endothelial Cell Cultures: Using porcine pulmonary artery endothelial cells (PPAECs), pre-treatment (50ug/mL) and post-treatment with rhMG53 (10 and 50ug/mL) reduced ET-1 production in supernatant as compared to untreated control (Figure 11A). When cells were lysed, Western blot analysis showed that treatment before H/R (pre-treatment) with 50ug/mL of rhMG53, but not treatment after hypoxia and before reoxygenation (post-treatment), resulted in significantly decreased ET-1 production within cells (Figure 11B). Next, studies with fluorescent conjugated Alexa647-rhMG53 were conducted and it was found that the H/R experiment in PPAECs led to rapid uptake of Alexa647-rhMG53 into the cytosol (Figure 11C) and was not restricted to the plasma membrane.

MG53 Administration During Rodent Transplantation Prevents Endothelial Injury:

With the goal of providing evidence that administration of exogenous human recombinant MG53 (rhMG53) is efficacious in mitigating damage to the endothelium in marginal donor allografts, rodent single lung transplantation after a period of 1 hour of warm ischemia was conducted. Prior to reperfusion of the transplanted allograft, the recipients were treated systemically with either rhMG53 (1 mg/kg) or saline (Figure 12A). Following a 3-hour reperfusion period, recipients who received rhMG53 had a significantly lower wet-to-dry ratio of the transplanted lobe, indicating less edema formation within the lung parenchyma (Figure 12B). At a molecular level, rhMG53 administration during transplantation was associated with a decrease in big endothelin-1 (Big ET-1) and its active form, ET-1, which is a known marker of endothelial damage. This decrease was seen in the plasma following the reperfusion period (Figure 12C, 12D). When cells were lysed, densitometric quantitation showed that treatment with rhMG53 (1 mg/kg) significantly reduced ET-1 levels in the tissue of the transplanted lobe (Figure 12E, 12F).

rhMG53 Mitigates Endothelial Injury and Preserves Lung Function Ex Vivo: Next, rhMG53 ability to confer endothelial protection was evaluated, and what effect this would have physiologically, in an ex vivo model utilizing ex vivo lung perfusion (EVL) as an assessment platform (Figure 13A). Rodents were assigned to 1 of 3 treatment groups: (1) no (0h) ischemic injury prior to 2h of perfusion with control perfusate; (2) 1h of warm ischemic time prior to 2h of

EVLP with control perfusate; and (3) 1h of warm ischemic time prior to 2h of EVLP with rhMG53 perfusate.

Perfusate was collected during EVLP at 0-, 30-, 60-, and 120-minutes and was analyzed for markers of endothelial, and cell membrane damage. Composite ET-1+ Big ET-1 was significantly increased in lung allografts which underwent ischemic injury prior to reperfusion, which was mitigated by addition of rhMG53 to the perfusate (Figure 18). Big ET-1 release was significantly mitigated by the addition of rhMG53 during all time points (Figure 13B), while the active ET-1 release was significantly decreased at 60- and 120-minutes (Figure 13C). To examine overall cell lysis, lactate dehydrogenase (LDH) was examined, which was significantly decreased in the treatment group after 120-minutes (Figure 13D). Finally, to analyze exposed basement membranes, hyaluronic acid (HA) release was measured as well. Again, rhMG53 treatment conferred protection, and the treatment group had significantly lower HA release at 120-minutes (Figure 13E).

From a physiologic standpoint, PaO₂ was significantly decreased in both the no injury and injury without treatment group, while this difference was not seen in the rhMG53-treated group (Figure 13F). Delta PaO₂, i.e., the change in oxygenation from the pulmonary artery canula compared to the left atrium canula, a significant decrease was seen in the injury group, however not noted in the rhMG53 group or no injury group (Figure 13G). Pulmonary vascular resistance (PVR) was significantly decreased in all groups at 120-minutes (Figure 13H), while pulmonary artery (PA) pressure was significantly increased in all groups at the end of the perfusion period (Figure 18). Wet-to-dry ratio showed no significant differences showing that any edema caused by the 120-minutes perfusion did not differ among groups (Figure 18).

After the 2h perfusion period, tissue analysis via hematoxylin and eosin (H&E) histological staining of lung sections showed that lungs without rhMG53 treatment sustained disruption of tissue architecture and developed parabronchial thickening that was mitigated by rhMG53 administration in the perfusate (Figure 14A). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of lung sections indicated that apoptosis was also increased by ischemic injury and was mitigated by rhMG3 (Figure 14B). Similarly, caspase 3/7 activity, as a biochemical indicator of apoptosis, was increased by ischemic injury and furthermore mitigated by rhMG53 treatment (Figure 14C). Fluorescent microscopy of lungs sections after the 2h perfusion period demonstrated that rhMG53 is effectively delivered to the injured lungs by EVLP (Figure 15A). After quantifying the degree of MG53 fluorescence, both the 1h WI and 1h WI+rhMG53 groups had significantly higher levels of MG53 expression compared to control (Figure 15B). Additionally, analysis of the rhMG53 in the perfusate showed that the accumulation

of rhMG53 in the lung tissue shown by microscopy was accompanied by a simultaneous reduction of rhMG53 in the perfusate over time (Figure 15C).

rhMG53 Mitigates Endothelial Injury and Preserves Lung Function in a Large Animal Transplantation Model: After demonstrating cellular protection and preserved physiologic function after administration of rhMG53 in small animal models, the hypothesis was tested in a large animal model. Pigs were assigned to treatment group or vehicle control, and after a period of 24-hours of cold static storage (CSS), a single left-lung transplant was performed. Pigs were treated with rhMG53 (1 mg/kg) or control (saline) by central access prior to reperfusion of the transplanted lobe (Figure 16A). All pigs that were administered rhMG53 were able to survive the full reperfusion period (4 hours), while 1 pig died at ~1 hour into reperfusion in the control group.

Gross pathology showed that the transplanted lobe (Figure Left) had less bullae formation, erythema, and injury when treated with rhMG53 compared to those which received vehicle (Figure 16B). From a physiologic standpoint, there was a significantly higher PaO₂ in the transplanted lobe in the treatment group (Mean (M): 445.2; Standard Deviation (SD): 55.5) compared to the vehicle group (M:201.9; SD: 182.4) at the end of experiment, while this difference was not noticeable in the native lobe (Figure 16C). The PaCO₂ of the transplanted lobe, native lobe and systemically was significantly lower in the treatment group compared to the control group (Figure 16D), while the pH was similarly significantly elevated in these corresponding lobes as well in the treatment group compared to the control group (Figure 16E).

At the end of the experiment, there was a significantly lower release of ET-1 in the bronchoalveolar lavage (BAL) of the rhMG53 treated pigs as compared to control (Figure 16F). Additionally, this release was significantly less in the plasma as well (Figure 16G). Tissue necrosis factor-alpha (TNF- α) and receptors of advanced glycation end products (RAGE), have been shown to regulate the release of ET-1, and each were significantly decreased in the transplanted lobe of the treatment group (Figure 17A, 17B). ET-1 mediates the release of monocyte chemoattractant factor-1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1), and interleukin-18 (IL-18). In the BAL at the end of the experiment, all cytokines had decreased release in the transplanted lobe's BAL (Figure 17C, 17D, 17E respectively). Additionally, to measure overall cell lysis, LDH was assessed in plasma samples and was significantly decreased at the end of the experiment (Figure 17F).

Discussion:

Here again rhMG53's ability to mitigate release of known markers of endothelial injury is shown, and furthermore preserve lung function during transplantation with exogenous administration. This investigation builds on the scientific premise that targeting the elemental process of cell membrane repair is a means to preserve the quality of the donor lung, thereby improving the outcome of lung transplantation. The data generated by this study supports the notion that rhMG53 has therapeutic benefit in protecting endothelial cells exposed to IRI and protect allograft lungs from further injury during EVLP and employed in lung transplantation to mitigate IRI improving early outcomes.

It was found that warm IRI to PPAECs causes elevation of detectable ET-1, and treatment with rhMG53 suppresses ET-1 elevation, as control BSA is ineffective in western blotting evaluation. Additionally, a significant portion of Alexa647-rhMG53 overlapped with FITC-AnnexinV, which is consistent with previous observations that rhMG53 recognizes exposed phosphatidylserine at the injured plasma membrane. Thus, the protective function of rhMG53 in endothelial cells may involve both plasma membrane and intracellular signaling. The rodent transplantation results indicated that rhMG53 administration preserves the endothelial barrier, as evident by a damped ET-1 release and decreased tissue edema, in the immediate reperfusion period following lung transplantation. Overall, by exploiting EVLP as a platform for lung evaluation and targeted therapeutic delivery, the potential therapeutic role of rhMG53 was evaluated. Administration of rhMG53 during the perfusion period mitigated release of known markers of endothelial and cell membrane integrity, as well as apoptotic response, demonstrating protection of this barrier. Furthermore, oxygenation was preserved throughout the perfusion period by the administration of rhMG53, demonstrating preserved physiologic function. Finally, analysis of rhMG53 in the perfusate showed accumulation of rhMG53 in the lung tissue, and simultaneous reduction of rhMG53 in the perfusate over time, which further supports that administration of rhMG53 is readily used by lung tissue to prevent further injury. Finally, the porcine transplantation model showed that rhMG53 administration decreased inflammation while preserving oxygenation and mitigating a respiratory acidosis.

Protection of the endothelium is paramount during lung transplantation. Disruption of this barrier allows for infiltration of immune cells which cause significant damage to the alveoli, leading to edema and poor allograft function. While this progression is mediated by numerous cytokines and molecular pathways, ET-1 plays a significant role. ET-1 is a potent vasoconstrictor and is released in response to hypoxia and inflammation. When the endothelium is injured, ET-1 is released which exerts its own effects on pulmonary vasculature and the alveoli. ET-1 increases

capillary permeability through the upregulation of vascular endothelial growth factor, as well as increases capillary hydrostatic pressure. When the endothelium is injured, ET-1 is released which exerts its own effects on the alveoli. ET-1 mediates nitric oxide release from endothelial cells, which signals epithelial cells in the alveoli to down regulate Na^+/K^+ adenosine 5'-triphosphatase which disrupts fluid clearance causing edema within the parenchyma. Additionally, the inhibition of ET-1 has been shown to decrease hypoxia induced apoptosis. All of these effects of ET-1 were readily mitigated throughout the experiments (as evident by directly decreasing edema or preserving oxygenation through presumably less alveolar edema), as well as downstream products of ET-1 to include: MCP-1, PAI-1, and IL-18.

The interaction of ET-1, RAGE and nuclear factor-kappa beta (NF-KB) is important to highlight. ET-1 expression can be regulated by a variety of factors, including RAGE. RAGE has been shown to mediate interference of membrane resealing of endothelial cells and promotes endothelial cell death and further damage to this membrane. When RAGE is activated in response to hypoxia, it causes nuclear factor-kappa beta (NF-KB) to translocate into the nucleus which promotes an inflammatory response and thus the release of $\text{TNF-}\alpha$ and in turn ET-1 expression and release. While the ability of MG53 to seal membranes is well documented, it can also suppress the release of NF-KB. The action of MG53 is multifactorial and confers protection through a variety of anti-pyrototic and necroptotic pathways, in which case MG53's ability to decrease ET-1 release involves the RAGE-NF-KB- $\text{TNF-}\alpha$ axis. The interference of this axis is evident throughout this study.

Damage to the endothelium serves as a significant contributor to lung edema, injury, and eventually PGD. The results support the premise that exogenous administration of rhMG53 to lungs during organ transplantation can mitigate endothelial damage by suppressing the release of ET-1. rhMG53 can mitigate ischemic induced lung injury shows a potential treatment for IRI, prevention of PGD and a novel therapeutic in the field of organ transplantation. These findings show that MG53 can aid and assist in resuscitating and repairing organs in a transplant setting.

Example 3: Mitsugumin-53 mitigation of ischemia–reperfusion injury in a mouse model.

The majority of donor lungs are discarded out of fear of PGD. Yet the specific mechanisms underlying lung injury and repair are poorly understood. This knowledge gap poses an obstacle to the development of novel lung transplant therapeutics. This study indicates that MG53 could function as a therapeutic to administer during transplantation to mitigate injury and improve outcomes.

Approximately 20% of donor lungs are able to be successfully transplanted, due to fears of organ quality negatively affected by hypoxia, edema, or trauma. Because of the inherent nature of

transplantation surgery, all organs have a period of ischemia on procurement from the donor and subsequently have a reperfusion injury when reimplanted in the recipient. This injury is known as ischemia– reperfusion injury (IRI). The end result of IRI at the cellular level is the destruction of the endothelium, loss of barrier protection, and migration of immune cells into the alveolar space causing further damage and edema. In the immediate postoperative period, this leads to poor organ quality and function, and can clinically manifest as primary graft dysfunction (PGD) in lung allografts. Development of PGD has profound short- and long-term impacts on transplant outcomes and results in significant recipient mortality and morbidity. Therefore, exploiting endothelial cell membrane injury repair pathways can offer novel means of salvaging marginal donor lungs by preventing IRI during transplantation.

Mitsugumin 53 (MG53) is a tripartite motif-containing family protein predominately expressed in skeletal muscle and alveolar epithelial cells. Upon tissue injury, it is released into the circulation to facilitate cell membrane repair. MG53's cell membrane repair capabilities are well described. In brief, when plasma membranes are damaged, MG53 senses the oxidized extracellular environment and binds to phosphatidylserine on membrane vesicles. It then traffics these vesicles to seal the damage membrane through a “plug and patch” method.⁷ Previous studies have shown MG53 can reduce acute lung injury, as well as offer cardioprotection to cardiac myocytes damaged by IRI. Modulation or administration of an exogenously produced recombinant human MG53 (rhMG53) and its related cell membrane repair is a potential novel therapeutic for the treatment of IRI and prevention of PGD associated with lung transplantation. It was hypothesized that exogenous administration of rhMG53 would confer endothelial protection and mitigate lung injury in a transplant-relevant in vivo model of IRI.

Methods:

C57BL/6J mice were subjected to 1 hour of ischemia (via left lung hilar clamp), followed by 24 hours of reperfusion. *mg53*^{-/-} mice were administered exogenous recombinant human mitsugumin-53 or saline before reperfusion. Tissue, bronchoalveolar lavage, and blood samples were collected at death and used to quantify the extent of lung injury via histology and biochemical assays.

Animals: All in vivo experiments used C57BL/6J (The Jackson Laboratory) MG53 knockout (*mg53*^{-/-}) mice weighing 20 to 30 g. A transgenic mouse line with genetically absent ability to secrete MG53 was generated by interrupting the MG53 DNA sequence as previously described. The founder mice were confirmed with absent levels of MG53 in blood circulation by immunoblotting. All animals were housed under standard conditions (humidity: 45%-70%,

temperature: 21 °C ± 3 °C, 12-hour light–dark cycle) and provided ad libitum access to water and standard diet. Animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee (Protocol #2012A00000135). All experiments were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory
5 Animals (NIH Publications No. 8023, revised 1978).

Hilar Clamp–Induced Ischemia–Reperfusion Injury Model: For IRI experiments, *mg53*^{-/-} mice were randomly assigned to treatment groups with rhMG53 or vehicle control (saline). Protocols for a mouse hilar clamp model have been described in detail. Briefly, when a surgical plane of anesthesia was met, mice (n ¼ 6/experimental arm) were intubated, and a left
10 anterolateral thoracotomy was performed. The left hilum was occluded using a small intubated, clamp (vein, artery, bronchus). The clamp was left in place for 1 hour. Before release of the clamp, vehicle or rhMG53 was administered to the mice via jugular vein injection (1 mg/kg). After allowing rhMG53 to circulate for 10 minutes, the vascular clamp was removed, and the left lung was allowed to reperfuse. Recruitment maneuvers were performed to expand the atelectatic lung
15 by increasing the positive end-expiratory pressure to 6 cmH₂O. The thoracotomy incision was then closed, and the mouse was subsequently awoken from anesthesia and transferred back to its housing. Standard postoperative pain medication was administered, and diet was resumed.

The mice were then kept alive for 24 hours under standard conditions. At death, the mice were prepped, anesthetized, and intubated as described earlier. A midline abdominal laparotomy
20 was performed, and 0.5 mL of blood was collected from the inferior vena cava. The mice were killed by cutting the inferior vena cava, and the thoracic cavity was exposed. A bronchoalveolar lavage (BAL) was performed by filling a syringe with 0.8 mL of sterile saline, injecting it into the endotracheal tube and withdrawing twice. The right and left auricles were cut, and the lungs were flushed by gravity through the pulmonary artery using an angiocatheter with 5 mL of
25 prechilled phosphate-buffered saline solution (GIBCO). The heart-lung block was then removed. The left lung was divided into 2 parts: The upper region was collected for snap-freezing, and the lower region was used for histology. Blood and tissue were analyzed for a previously described biometric profile of lung health.

Biochemical Assays: Colorimetry and fluorescence for all biochemical assays were
30 measured using the POLARstar Omega multi-mode microplate reader (BMG LABTECH).

Enzyme-Linked Immunosorbent Assay: Tissue homogenates were collected from the lungs. The levels of endothelin-1 (ET-1) (Enzo Life Sciences), hyaluronic acid (HA), tumor necrosis

factor alpha (TNF-a), and interleukin (IL)-1b (R&D Systems) were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Lactate Dehydrogenase Release Assay: Lactate dehydrogenase (LDH) release was measured in perfusate using the LDH cytotoxicity detection assay kit (Clontech Laboratories) according to the manufacturer's instructions. The optical density values were analyzed at 492 nm and normalized relative to plate reference absorbance at 620 nm and background LDH in control wells.

Adenosine Triphosphate Assay: Adenosine triphosphate (ATP) level in tissue was measured using the ATP Colorimetric/Fluorometric Assay Kit (Biovision), according to the manufacturer's instructions. Absorbance was measured (OD 570 nm) in a micro-plate reader.

Western Blot Analysis: Lung tissue samples were homogenized in RIPA lysis buffer (Millipore) with a 13-protease inhibitor (Cell Signaling Technology). Equal amounts of lysate proteins were electrophoresed on 6% to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking membranes in a Tris-buffered saline/0.1% Tween 20 supplemented with 5% nonfat dry milk for 3 hours. Membranes were incubated with primary mouse antibody against gasdermin-D (GSDMD). The membrane was incubated with horseradish peroxidase as a secondary antibody (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. Protein levels on the membrane were determined using Western Lightning Plus-ECL (PerkinElmer) and autoradiography film (Denville Scientific). Incubation with monoclonal mouse b-actin antibody (Sigma-Aldrich) was conducted for comparative control. Quantitative results were obtained using Image J software (NIH).

TUNEL Staining: Apoptotic cells in the lung tissue section were determined using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. The assay uses terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which identifies apoptotic cells by labeling DNA fragmentation sites. The tissue was also counterstained with DAPI to permit visualization of cell nuclei. After TUNEL staining, samples were cover-slipped using Prolong Diamond (P36966; Thermo Fisher Scientific) mounting medium. Tissues were imaged using a Leica Thunder 3D imaging system equipped with a Leica K5 fluorescence imaging camera. TUNEL and DAPI images were merged to allow visual identification of apoptotic cells using LasX V2.3.0 and ImageJ analysis software.

Immunohistochemistry Staining: Paraffin-embedded formalin-fixed sections of murine lung were cut from tissue blocks at 4- to 6-mm thickness and mounted on slides. Mounted tissue sections were heated at 60 °C for 30 minutes and washed 4 times in xylene each for 10 minutes to remove the paraffin wax, followed by rehydration through an ethanol series, before washing and storing in dH₂O until use. Antigen retrieval was conducted via 30 minutes of incubation in 100 °C sodium citrate buffer followed by preincubation in 1% fetal bovine serum/glycine blocking solution. A specific primary antibody against MG53 (1:200, Rabbit, Homemade) and CD31(1:200, Rat, 550,274, BD Pharmingen) was incubated with the tissue section overnight. The details for creation of homemade MG53 primary antibody have been described in detail. For the visualization, tissue sections were stained with Alexa Fluor 647 (Rabbit) and Alexa Fluor 555 (Rat) second antibodies (1:500, A32733, Invitrogen; 1:500, A21434, Invitrogen). The stained tissue sections were mounted in DAPI Fluoromount-G (0100-20, Southern Biotech). Images were taken using the Nikon A1R-MP Laser Scanning Confocal Microscope.

Histological Analysis: Histological samples were stored in 10% formalin and transferred to ethanol for paraffin embedding, and 5-mm sections were cut and processed. Hematoxylin–eosin and IHC staining were performed using standard methods.

Cellular Differential Staining: BAL fluid was centrifuged at 800g for 10 minutes, and the supernatant was removed. The cells were then resuspended in the appropriate buffer and automatic cell count was performed using Countess 3 (Invitrogen). The resuspended cells were diluted to an appropriate concentration and loaded into a cytopsin funnel, and again centrifuged at 800g for 10 minutes. The slides were then dried overnight at room temperature and stained with Differential Quick Stain Kit (Electron Microscopy Sciences) and analyzed with a light microscope for manual cell differential.

Statistical Analyses: In vitro experiments were repeated 3 times to test consistency and reliability, and representative data sets were displayed. The results were expressed as mean $\pm$ SD for each treatment group in each experiment. Significance was determined between 2 groups using a 2-tailed Student *t* test or among 3 or more groups using a 1-way analysis of variance with Dunnett's post hoc analysis to control for the familywise error rate associated with multiple comparisons of the experimental treatment groups with the untreated group. Analyses were performed using GraphPad Prism 7 software.

Results:

Administration of recombinant human mitsugumin-53 showed a significant decrease in an established biometric profile of lung injury as measured by lactate dehydrogenase and endothelin-1 in the bronchoalveolar lavage and plasma. Biochemical markers of apoptosis and pyroptosis (interleukin-1b and tumor necrosis factor-a) were also significantly mitigated, overall demonstrating recombinant human mitsugumin-53's ability to decrease the inflammatory response of ischemia-reperfusion injury. Exogenous recombinant human mitsugumin-53 administration showed a trend toward decreasing overall cellular infiltrate and neutrophil response. Fluorescent colocalization imaging revealed recombinant human mitsugumin-53 was effectively delivered to the endothelium.

Mitigation of Ischemia-Reperfusion Injury as Assessed by Established Makers of Lung Injury and Endothelial Cell Integrity: After reperfusion and survival, LDH expression was significantly lower in the plasma and BAL of the mice that underwent treatment with rhMG53 (Figure 20A, $P < .05$). Additionally, ET-1 was significantly lower in the mice that received treatment compared with vehicle in both BAL and plasma (Figure 20B, $P < .05$). Finally, there was no significant difference of HA release in the treatment versus the control group (Figure 20C).

Impact of MG53 on Lung Cellular Infiltrate in Response to Ischemia-Reperfusion Injury: In BAL samples, there was a trend toward reduction of total cell count, but this difference was not significant (Figure 21A). Manual cell differential of the BAL samples fixed to slides revealed attenuation of neutrophil infiltration in the rhMG53-treated group compared with vehicle, but this was not significant (Figure 21B). Macrophage presence did not significantly differ between groups (Figure 21C). In the rhMG53-treated group, there was significantly less neutrophil infiltration when compared with macrophages (Figure 21D); however, this was not true of the saline-treated group (Figure 21E).

Inflammatory, Apoptotic, and Pyroptotic Changes After rhMG53 Administration: TUNEL staining of lung sections indicated that apoptosis was also increased by IRI and mitigated by rhMG53 administration (Figure 22A). The degree of apoptosis in TUNEL staining was further quantified using Gene 5 software (Agilent Technologies). This also showed a significantly higher amount of apoptosis in saline-treated mice compared with rhMG53 treatment (Figure 22B). Biochemical markers of apoptosis and pyroptosis assessed included IL-1b, TNF-a, full-length GSDMD, and cleaved GSDMD. In the plasma, IL-1b was significantly decreased in the treatment group when compared with control (Figure 23A), as well as the BAL fluid (Figure 23B).

TNF- α showed a similar pattern and was significantly delivered to lung tissue, as demonstrated by the MG53 staining. In the rhMG53 treatment group, co-localization of MG53- and CD31-stained cells showed that rhMG53 effectively delivered the endothelium, that is, the site of injury.

Hematoxylin–eosin staining of lung sections showed that compared with the MG53 treatment group, animals subjected to IRI with vehicle alone demonstrated increased signs of tissue damage, disruption of tissue architecture, and peribronchial thickening (Figure 25). Finally, the degree of MG53 fluorescence colocalization images was further quantified, using Gene 5 software (Agilent Technologies). This also showed a significantly higher amount of MG53 in lung tissue in rhMG53-treated mice compared with vehicle treatment.

10 Discussion:

This study builds on the scientific premise that targeting the elemental process of cell membrane repair is a means of preserving endothelial integrity and mitigating IRI. The data generated by this study supports the notion that MG53 is an integral part of the cell membrane repair machinery and has the potential to be a novel therapeutic decrease in the serum (Figure 23C) as well as BAL fluid (Figure 23D). Western blotting showed more expression of full-length and cleaved GSDMD (Figure 24A). Densitometric analysis showed significantly less full-length GSDMD (Figure 24B) and cleaved GSDMD (Figure 24C) in tissue homogenate as well. Additionally, ATP was measured as a surrogate of mitochondrial function and pyroptosis. Mice treated with MG53 had significantly higher levels of ATP (Figure 24D) found in their tissue.

Fluorescent microscopy of lung tissue sections after IRI stained for MG53, CD31, DAPI, and the resulting merged image is shown in Figure 25A. Imaging demonstrated that *mg53*^{-/-} mice treated with vehicle had no background MG53 expression. MG53 and CD31 co-localization in the saline-treated group demonstrated no overlap of MG53 and CD31. When treated, rhMG53 was effectively agent in lung transplantation.

At the cellular level, IRI in lung tissue results in endothelial dysfunction and injury, manifesting as a recruitment of immune cells into the injured parenchyma and airway and resultant alveolar edema. The classic cell type associated with early IRI-induced PGD is the neutrophil. Neutrophils invade the parenchyma and alveolar space early during IRI leading to exacerbated lung injury and inflammation. In addition to causing direct oxidative tissue, neutrophils amplify the proinflammatory response through interactions with a multitude of immune cells. This neutrophilic inflammation is particularly damaging in the transplantation setting due to enhancement of the adaptive immune response increasing the potential for graft rejection. The benefit of blocking neutrophil activation during reperfusion has been shown to be protective against

IRI in lung allografts. The ability to hamper neutrophil recruitment and response after reperfusion is a way to prevent worsening IRI. rhMG53 administration attenuated the cellular response in the alveolar fluid and mitigated neutrophil migration from IRI in alveolar fluid.

In addition to mitigating inflammatory cellular infiltrate to the lungs, administration of rhMG53 resulted in an overall reduction in known biomarkers of cellular injury and death. Clinically, biometric profiles are predictors of PGD and give the clinician objective data when diagnosing and treating adverse outcomes in the immediate postoperative phase. IRI is a complex process mediated by a multitude of damage-associated molecular patterns (DAMPs) and inflammatory signaling cascades that lead to impaired cell function, resulting in endothelial cell death. During this process, markers of inflammation and cell damage are released and can be quantified in the systemic circulation. TNF- α is common proinflammatory cytokine that is upregulated downstream by DAMPS. In this study, TNF- α was significantly decreased in the serum as well as BAL fluid. ET-1 is a marker of endothelial cell integrity during lung injury and has both mitogenic and proinflammatory effects that are implicated in the pathophysiology of IRI. Clinically ET-1 is a prognostic biomarker, and elevations of serum ET-1 have been shown to correlate with the development of PGD. ET-1 release was significantly mitigated by administration of MG53 compared with control in the BAL and serum of mice subjected to IRI. Likewise, cellular release of LDH is a widely recognized marker of cell membrane compromise, oxidative stress, and damage. In transplantation models, LDH has been found at substantially lower levels in perfusate of lungs that are considered suitable for transplantation after ex vivo lung perfusion. Furthermore, post-transplantation elevation of serum LDH is an early clinical indicator of acute rejection. Administration of exogenous MG53 was able to mitigate oxidative stress in mice with IRI as well. HA is a marker of acute lung injury, and during transplantation can be seen elevated in patients with acute rejection. In this study, there was a nonsignificant trend in reducing HA release in both the BAL and plasma through the administration of rhMG53.

There are 2 major pathways that contribute to cell death after IRI: apoptosis and pyroptosis. Apoptosis is a form of programmed cell death that is triggered in response to hypoxia during IRI. Cells induce death via proteolysis from caspases. This form of injury contributes to IRI during the early phases of allograft reperfusion. Apoptosis is most severe after reperfusion, and prior studies have noted induction of apoptosis as early as 30 minutes after reperfusion. Quantification of apoptosis was evaluated by TUNEL staining, which demonstrates a decreased intensity in animals that were treated with rhMG53. In this work, apoptotic cell death was observed as long as 24 hours after IRI. Pyroptosis is another form of programmed cell death and is more often associated with IRI than apoptosis. It differs from apoptosis in that cell death arises from plasma membrane

disruption, organelle swelling, and mitochondria dysfunction. Injury and formation of DAMPs are recognized by NOD-like receptors, which results in the formation of inflammasomes. Inflammasomes recruit and subsequently turn pro-caspase 1 into its active form. Activated caspase 1 splits full-length GSDMD to cleaved GSDMD and activate IL-1b, initiating pyroptosis. During pyroptosis, cleaved GSDMD interacts with cell-membrane lipids to form transmembrane pores, which allows IL-1b, additional cytokines, and signals to be released and promote further inflammation and cell death. This step in the IRI model was significantly suppressed by the administration of rhMG53 and was further confirmed by the mitigation of IL-1b release. For a surrogate for overall cell viability and mitochondria function, ATP was measured in tissue homogenate. When pretreated with MG53, ATP was significantly higher in tissue when compared with control. These cells with a high amount of ATP indicate functional respiration and intact mitochondrial function.

Our previous observations have demonstrated that MG53 is involved in cell membrane repair and machinery. Protection of the endothelium is paramount in lung transplantation because damage to this barrier leads to further edema, injury, and eventual failure of the allograft. The delivery of therapeutics to the endothelium is vital to any candidate treatment intended to mitigate IRI, because the endothelium is the barrier between the recipient blood and the donor organ. IHC imaging after IRI and subsequent 24-hour survival demonstrated a co-localization of MG53 and CD31. CD31 is a transmembrane immunoglobulin-type inhibitory receptor that is expressed on endothelial cells. Fluorescent imaging of lung tissues in animals administered rhMG53 showed that rhMG53 is observed in the lungs and co-localizes with CD31 positive endothelial cells. This observation indicates that the exogenously supplemented MG53 protein is locally bioavailable in the lung tissue and specifically associated with the damaged endothelium. The continued detection of MG53 in the *mg53*^{-/-} background mice after 24 hours of reperfusion demonstrates sustained presence in the lung environment. The genetic ablation of MG53 resulting in a significant injury that is ameliorated by exogenous rhMG53 demonstrates the basis of a novel therapeutic to enhance lung transplantation.

Conclusions:

Collectively, these data demonstrate that purified rhMG53 is protective against IRI-mediated cellular injury and that systemically administered rhMG53 localizes to the lung endothelium to provide sustained protection against cell damage and death. The mechanism of protection is likely multimodal, including increased maintenance of cellular integrity, reduction in primary and secondary inflammatory tissue damage, reduction of oxidative stress, and reduced

induction of programmed cell death. Endothelium damage and resultant proinflammatory signaling is a significant contributor to lung edema, injury, and eventually PGD. These results show that MG53 can mitigate lung injury caused by IRI. This is the first report of MG53 offering a therapeutic benefit in lung transplant-relevant IRI.

5 Example 4: Mitsugumin-53 regulates pyroptosis and cell integrity during lung transplantation.

Lung transplantation was the only definitive therapy for end-stage lung disease. While the waiting list of patients eligible for transplant grows, the number of suitable donor organs falls far short of the demand. Less than 20% of offered lungs are successfully transplanted due to a limited
10 ischemic time, and poor donor lung quality, manifested by pulmonary edema, hypoxia, or trauma. To address the critical unmet demand for suitable donor allografts, attention is directed to donation after circulatory death (DCD) and marginal or extended criteria donors. Unfortunately, ischemia-reperfusion injury (IRI), which happens universally in transplantation, is especially pronounced in these allografts. Clinically, this circulatory death process with variable warm ischemia leads to
15 worsening IRI and donor organ injury which is manifested as severe primary graft dysfunction (PGD). The inflammatory process associated with PGD leads to the caspase-1 dependent pyroptotic cell death (inflammatory cell death) which releases cytokines, lysosomes, and vesicles, tied to PGD. Long-term recipients with PGD are at higher risk of developing chronic lung allograft dysfunction (CLAD) which leads to early graft failure and poor long-term survival. Out of fear of
20 adverse outcomes associated with PGD, the transplant community discards ~80% of the potential donor allografts.

The knowledge gap in understanding the molecular mechanisms driving pyroptosis associated with IRI is an obstacle to expanding lung transplantation. Of critical importance: (1) IRI happens universally in transplantation, triggering inflammatory responses and cell death which
25 leads to PGD, organ rejection, and increased mortality; (2) IRI contributes to pyroptosis and release of inflammatory cytokines; (3) maintaining endothelial cellular integrity prevents IRI; and (4) MG53 is a novel factor to regulate pyroptosis, preserving cell integrity. To support this competitive renewal, it has been shown that genetic ablation of MG53 (*mg53*^{-/-} mice) leads to increased susceptibility of the lung to stress-induced injuries and that overexpression of MG53
30 (tPA-MG53 mice) leads to protection. First, this study established that the rhMG53 can prevent and protect lung injury in rodent and large animal models. Second, it was discovered that a significant component of the lung IRI in transplantation is manifest at the endothelial cell level and that repair of the endothelial membrane through rhMG53 blocks endothelin-1 release

improving lung function. Third, these therapeutic benefits were expanded into inflammasome modulation. It was identified that MG53 influences inflammation by modulating NLRP3, reducing pro-inflammatory cytokines. Finally, in addition to membrane repair function, it was found that MG53 inhibits transplantation induced inflammation via suppressing pyroptosis of immune cells.

5 Based on these data, it was hypothesized that lung IRI induced endothelial cell disruption and immune cell pyroptosis are important events leading to lung transplant failure where MG53 plays dual roles in promoting lung tissue integrity and inhibiting post-transplant inflammation to improve success of lung transplantation.

The mechanism of MG53 in preservation of lung endothelial cell integrity in
10 **transplant related injury.** Cellular and molecular imaging tools are used to derive a mechanism for how circulating MG53 preserves the endothelial integrity, improves cell survival and reduces inflammation during IRI. Mechanisms are determined by which pulmonary vascular endothelial cells contribute to leukocyte signaling and inflammation. Furthermore, precision cut lung specimens allow modeling of endothelial cell-to-tissue level interaction and potential benefit of
15 these therapies.

The role of MG53-mediated pyroptosis inhibition to preserve immune cells survival after IRI, mitigating intercellular signaling with potential therapeutic benefit in lung transplantation. This step focuses on the role of MG53 in regulating transplantation induced pyroptosis. The therapeutic benefits of MG53 to repair cell membrane damage induced by GsdmD-
20 N mediated membrane pore formation are evaluated. *mg53*^{-/-}, tPA-MG53, *GsdmD*^{-/-} and WT mice are used to determine the role of MG53 in post-IRI induced neutrophilic pyroptosis. The effective dose and time window of rhMG53 to protect neutrophils against pyroptotic death is determined. Furthermore, lung transplantation experiments are performed in neutrophil specific *GsdmD*^{-/-} mice to determine the role of pyroptosis in an *in vivo* model.

25 **Large animal and human lung *ex vivo* lung perfusion (EVLP) with rhMG53 to preserve and rescue lung allograft integrity.** The therapeutic benefit of rhMG53 supplementation for pulmonary applications is established. Expertise in EVLP and transplantation is used to test the efficacy of rhMG53 in rescuing the injured allograft procured from pigs and non-transplantable human donor lungs.

30 This study increases the mechanistic understanding of intrinsic lung donor allograft biology and integral role of pyroptotic cell death in response to IRI which results in PGD. These studies provide the basis and rationale of a translational trial approach of rhMG53 intervention in subsequent investigations.

IRI induces primary graft dysfunction: No cure exists for patients with end-stage lung disease and lung transplant is the only effective treatment strategy. The transplant waiting list grows each year though the number of transplants performed annually has stagnated at ~2,000/year since 2010. The lack of quality lung donors is limiting with only 17% of donated lungs successfully being transplanted. Of those patients fortunate enough to receive a transplant, 32% develop severe grade 3 primary graft dysfunction (PGD). PGD detrimentally impacts patient outcomes and survival. Ischemia reperfusion injury (IRI) drives PGD, whose hallmark is inflammation, leucocyte infiltration, and the activation of cellular immune responses that impact long term survival (Figure 26). The lung transplant community discards ~80% of potential donor allografts out of fear of PGD. PGD leads to pulmonary vascular endothelial cell injury (Figure 27) and increased vascular permeability, which results in increased extravascular water/edema and respiratory failure. Thus, as it is difficult to predict PGD and it leads to significant adverse outcomes, agents providing effective mitigation of inflammation and PGD are a novel and of therapeutic benefit. There are no Food and Drug Administration approved treatments to heal lung endothelial and epithelial barrier functions.

Pyroptosis is critical for the inflammatory response to IRI: Pyroptosis, a lytic form of cell death, is a key pathway triggering inflammation. Upon activation (e.g., danger associated molecular patterns (DAMPs) induced by IRI and factors associated with reperfusion), caspase-1 processes IL-1 β from an inactive precursor to the active form. Caspase-1 also cleaves Gasdermin D (GsdmD), a key factor of pyroptosis pathway. The cleaved GsdmD (GsdmD N-terminus or GsdmD-N) forms transmembrane pores that lead to cell membrane injury and leakage of intracellular contents, including active IL-1 β , which triggers cell death and tissue inflammation. Although endosomal sorting complexes required for transport (ESCRT) machinery has been proposed to repair pyroptosis-induced membrane damage, ESCRT blockage does not cause 100% pyroptotic cell death showing that there is an alternative pathway(s) for membrane repair after GsdmD activation.

MG53 protects against IRI induced lung cell membrane damage: Repair of injury to the cell membrane is an important aspect of physiology, and inadequate membrane repair results in pathophysiology in many human diseases, including cardiovascular dysfunctions. MG53 is an essential component of the cell membrane repair machinery. MG53 functions in vesicle trafficking and allows for nucleation of intracellular vesicles at sites of membrane disruption. Genetic ablation of MG53 results in defective membrane repair. The benefit of MG53 in lung injury and explored the therapeutic benefit in transplantation and acute lung injury has been demonstrated. Application

of rhMG53 protein reduced pyroptotic cells in IRI injured lung tissue (Figure 33). Furthermore, live cell imaging revealed RFP-MG53 translocates to cell membrane upon activation of pyroptosis (Figure 36). *In vitro* biochemical study also revealed that MG53 could bind to GsdmD (Figure 37A) and inhibit its cleavage by caspase-1 (Figure 37B).

Of critical importance: (1) IRI happens universally in transplantation triggering robust inflammatory responses and cellular death which leads to PGD, organ rejection, and increased mortality; (2) IRI contributes to pyroptosis activation and release of inflammatory cytokines; (3) maintaining endothelial cellular and cell junction integrity prevents IRI; and (4) MG53 is a factor that regulates pyroptosis, preserving cell integrity (Figure 28). It has been shown that an absence of MG53 in mice leaves the lung at risk of injury. It was demonstrated that rhMG53 protein can mitigate lung dysfunction associated with ventilator induced lung injury (VILI) and IRI. A rat model of EVLP was established and it was found that exogenous rhMG53 in the lung perfusion solution can protect against injury to endothelial cells. In rodents and humans, circulating MG53 is normally present at low levels. The injury, activation and subsequent dysfunction of neutrophils and the interaction with vascular endothelial cells central to the pathophysiology of IRI and PGD (Figure 27).

Numerous aspects of this study and the role of pyroptosis in lung transplantation have not been well defined. Further, it was found that neutrophils undergo pyroptosis and contribute to IRI damage. This is a new and crucial role of neutrophils to IRI induced lung damage, which also serve as a drug target to protect the lung allograft. Herein disclosed are novel findings that link MG53-mediated cell membrane protection to control activation of pyroptosis during IRI injury. The molecular machinery that governs the functional interactions between MG53 and GsdmD to regulate pyroptosis has significant implications for lung IRI research. Furthermore, the animal models, such as neutrophil specific *GsdmD*^{-/-} mice, murine and porcine lung transplantation have been established to study the role of pyroptosis in lung transplantation. Finally, native MG53 protein is available in circulation and is released in significant quantity to neighboring cells upon tissue injury. These findings are critical, as they not only support the physiology of MG53 in lung but also show the safety for the use of rhMG53 protein to prevent PGD after lung transplantation.

Experimental models: Rodent and porcine large animal models of EVLP and the corresponding *in vivo* models of lung transplantation have been established (Figures. 35, 39, 41, 43 & 44). The rodent lung transplant models have allowed evaluation of the *in vivo* function of MG53 on the progression of transplant associated lung injury. It has been recapitulated the

transplant effect of IRI using precision cut lung slices (PCLS) to simulate the transplant experience inhibition in tissue preserving the complex 3-dimensional micro- environment of the lung.

The mechanism of MG53 in preservation of lung endothelial cell integrity in transplant related injury: The potential benefit of MG53 is shown at the endothelial cell level, cellular, molecular, and live cell imaging tools are used to derive a mechanistic base for how circulating MG53 preserves endothelial cell integrity, improves cell survival, and reduces inflammation during IRI. These studies determine mechanisms by which pulmonary vascular endothelial cells contribute to leukocyte signaling and inflammation. This study establishes a set of biomarkers modulated by rhMG53 that quantifiably predicts lung injury prior to transplantation.

rhMG53 suppresses IRI-induced ET-1 elevation in rat lung and cultured endothelial cells: Utilizing this established rat model of EVLP, the impact of rhMG53 on lung injury was evaluated. It is demonstrated (Figure 29A) that 1 hour of warm ischemia induced structural damage to the rat lung that could be rescued by EVLP with exogenous rhMG53 (5 µg/ml perfusate concentration). Prior studies by other investigators have shown that ET-1 correlates with PGD and lung injury. ET-1 is elevated in transbronchial biopsies under suspicion of infection or rejection post lung transplantation. It was found that warm ischemic injury to the rat lung causes elevation of ET-1 in a time- dependent manner in the perfusate, and EVLP delivery of rhMG53 (5 µg/ml) suppresses ET-1 elevation (Figure 29A). The study has been able to utilize ET-1 as a measure of lung injury in BAL. Immunohistochemistry (IHC) imaging showed that ischemic lung tissue contains elevated levels of ET-1 compared with control (no ischemia), whereas rhMG53 administration reduces ET-1 intensity in the injured lung tissue. These findings support that rhMG53 delivered to the allograft via EVLP can modulate ET-1 expression preserving lung integrity and quality.

rhMG53 protects against injury to endothelial cells: Live cell imaging was performed with GFP-MG53 transiently expressed in aortic endothelial cells (AoEC). Following micro-electrode induced injury to the plasma membrane, rapid translocation of MG53 was observed at the injury site, similar to what has been previously observed with other cell types. Using the established glass-beads induced membrane damage assays, it was showed that rhMG53 displayed dose-dependent reduction of LDH release, supporting the membrane repair function of rhMG53 in AoEC and pulmonary artery endothelial cells (PAEC). Moreover, it is shown that rhMG53 is effective in protection of H₂O₂ and IRI induced endothelial cell injury.

Studies were conducted with fluorescent conjugated Alexa647-rhMG53, and it was found that the hypoxia and reoxygenation (H/R) induced injury to AoECs led to rapid uptake of Alexa647-rhMG53 into the cytosol (Figure 30). A significant portion of Alexa647-rhMG53 overlapped with FITC-Annexin V, which is consistent with a previous observation that rhMG53 recognizes exposed phosphatidylserine (PS) at the injured plasma membrane. As control, Alexa647-BSA did not show uptake into the cells. Thus, the protective function of rhMG53 in endothelial cells may involve both plasma membrane and intracellular signaling.

Damage to the endothelium serves as a significant contributor to lung edema and injury. These data support that rhMG53 is an effective reagent to preserve endothelial cell integrity. This study aimed to define the mechanistic function of circulating MG53 or exogenous rhMG53 in preservation of endothelial cell integrity. These studies test the hypothesis that, in addition to plasma membrane preservation, endocytic uptake of rhMG53 can contribute to the survival of endothelial cells under ischemic conditions that occur during lung transplantation. The data shows a candidate pathway is the VEGF receptor (Figure 31). Live-cell imaging and CRISPR-gene editing are utilized to show the mechanism of VEGFR2-mediated MG53 uptake in endothelial cell protection.

MG53 endocytic uptake to preserve endothelial cell integrity: It was previously shown that extracellular rhMG53 can recognize exposed PS to preserve plasma membrane integrity of lung epithelial cells under A/R conditions. To test if MG53 binding to PS mediates membrane repair function of endothelial cells, experiments are conducted by co-treating the hypoxic cells with different lipids (PS and phosphatidylcholine) and rhMG53 to test whether competitive binding with the cell membrane disruption would facilitate or inhibit rhMG53-mediated endothelial membrane repair. In a co-culture model of endothelial cells and THP-1 macrophages, the response to H/R results in an exacerbated inflammatory and VEGF response. It was hypothesized that receptor-mediated endocytosis will facilitate MG53's entry into endothelial cells to modulate their response to hypoxic injuries. Toward identification of a putative membrane receptor for rhMG53, a combination of biochemical, molecular, and Laser Capture Micro-dissection (LCM) methods was used and VEGFR2 was identified as a potential receptor for MG53 on the plasma membrane of stem cells. CRISPR was employed to knockout VEGFR2 from mesenchymal stem cells (MSCs) (Figure 31A) and live cell imaging to assay the impact of VEGFR2 knockout on rhMG53 uptake by MSCs. As shown in Figure 31B, CRISPR-VEGFR2 treated cells (CRISPR construct expresses GFP driven by separate promoter) failed to show rhMG53, while uptake of rhMG53 is robust in MSCs either without CRISPR-VEGFR2 transfection (non-GFP cells in

Figure 31B, left) or control CRISPR transfections (Figure 31B, right). Together, these data show that VEGFR2 is a candidate receptor to likely mediate rhMG53 endocytosis into MSCs (Figure 31C). To test the role of VEGFR2 in facilitating endocytic uptake of rhMG53, first it was examined whether VEGFR2 expression is altered during hypoxic treatment of PAEC and AoEC.

5 The rationale being that PAECs function in a relative hypoxic physiologic environment as compared to AoECs and would potentially respond to transplant related hypoxia and IRI differently. Published studies from Ulyatt *et al.* and Nadeau demonstrate that hypoxia decreases VEGFR2 expression showing a role of VEGFR2 in the lung under hypoxic, transplant related conditions. It was proposed to use CRISPR to knockout VEGFR2 from
10 PAEC and AoEC to test the contribution of VEGFR2 mediated rhMG53 uptake into endothelial cells, and its impact on rhMG53-mediated preservation of endothelial cell integrity and survival under hypoxic conditions. Complementing the CRISPR-gene editing approach, pharmacological agents that influence endocytosis or pinocytosis pathways for rhMG53 entry into AoEC and PAEC (i.e., receptor mediated via clathrin coated pits or fluid phase mediated via
15 non-clathrin coated endosomal uptake across the cell membrane) are used.

Mechanism of endothelial cell to macrophage signaling and the role MG53 inhibition in mitigating IRI response: Published studies have shown that ET-1 expression is controlled by numerous stressors on endothelial cells (e.g., cytokine, oxidative stress, hypoxia shear) and increased ET-1 levels are modulated by hypoxia or cytokine induction of endothelin converting
20 enzyme (ECE) activity to cleave the precursor big ET-1. The role of ET-1 as a marker of lung injury or rejection post-transplant has been well established. Here the study explores the relationship between ET-1 and VEGFR2 mediated endocytic uptake of MG53 in protection against lung injuries. Endothelial cell IRI induces generation of reactive oxygen species (ROS) which may contribute to elevation of ET-1. It has previously been demonstrated that rhMG53 can enter cells,
25 protect mitochondria from injury, and reduce ROS generation.

The pulmonary vascular endothelium is the gateway to leukocyte infiltration in the lung parenchyma: THP-1 macrophages were isolated with *mg53*^{-/-} genetic ablation and through co-culture with endothelial cells and when the co-culture model of where *mg53*^{-/-} THP-1 cells are utilized in a H/R model, increased inflammasome response was demonstrated (Figure 32, IL-1 β ,
30 TNF- α , LDH) which is mitigated by administration of rhMG53. To elucidate the contribution of ROS to ET-1 release with endothelial cell injury, ROS and rhMG53 uptake into PAEC and AoEC cells are simultaneously monitored, and tests are performed to find whether rhMG53-mediated suppression in ROS correlates with reduction of ET-1 release from these cells. These

studies test the possibility that rhMG53 can protect mitochondrial dysfunction, to reduce ROS for the suppression of ET-1.

Precision Cut Lung Slices (PCLS) with murine and human lungs undergoing IRI:

While cell culture (Figures. 30-34) models have the ability to provide insight into mechanistic approaches to the MG53 protection of endothelium and the ability to regulate inflammation and indirectly regulate cell survival, there are limitations. To simulate the transplant episode, non-transplantable human tissue and diseased tissue are obtained from a local organ procurement organization (Lifeline of Ohio OPO) and a transplant repository. PCLS undergo intervals of H/R with the addition of dose escalating rhMG53, disulfiram (as pyroptotic inhibitor control) and the combination. This disease modeling allows the assessment of the secretome, cytokines and environmental stressors as well as the interaction of the micro-environment with the endothelial cells, leukocytes, and pneumocytes. Supernatant is collected for analysis and to be used as conditioned medium for cell culture and analysis. Advanced imaging (Leica Thunder) is utilized to perform high resolution imaging and time lapse microscopy. Additionally, Spatial Transcriptomics via Visium (10x Genomics) is employed as a functional discovery system to analyze whole transcriptomes in healthy and IRI of non-transplantable human donor lung sections. Application of this methodology in Visium data sets from distinct anatomical regions of donor control and IRI lungs to reveal distinct transcriptomic profiles in spatial clusters of alveolar and airway regions and high accuracy in capturing the architecture. Analyses are performed to determine the cell composition of transcriptional clusters and determination of gene signatures to identify and locate transitional cell types and states in lung disease and their cell molecular signatures, followed by high-resolution and confirmatory studies using imaging and spatial studies with Xenium technology.

The role of MG53-mediated pyroptosis inhibition to preserve immune cells survival after IRI, mitigating intercellular signaling with potential therapeutic benefit in lung transplantation.

Activation of pyroptosis happens quickly after lung IRI (Figure 33): Activation of different cell death pathways following IRI was determined. It was found that when activation of pyroptosis is tested by cleavage of GsdmD (GsdmD-N) at different time points following IRI, GsdmD-N showed that pyroptosis happened quickly, 90 mins, after IRI and persisted at least 3 days, showing a role of pyroptosis in the acute phase of lung IRI.

Neutrophils undergo post-IRI pyroptosis that is inhibited by rhMG53 treatment:

As IRI induces massive cell death in lung tissue, It was first tried to determine what cell type(s) in the lung parenchyma underwent pyroptosis following IRI. The first observation was endogenous lung cells. Thus, epithelial cells were treated with lipopolysaccharide (LPS) and Nigericin (Ni), an established protocol to induce pyroptosis in immune cells. It was observed that LPS+Ni failed to induce pyroptosis in epithelial cells. As immune cell infiltration happens after IRI, next checked was whether pyroptosis happens in immune cells. Mouse lung tissue slides were stained with CD68 (a macrophage marker) and MPO (a neutrophil marker). Indeed, it was found that majority of IRI induced pyroptotic cells are neutrophil, but not macrophage (Figure 34A). More interestingly, when rhMG53 treated lung slides were stained, it was found that both total pyroptotic cells (Figures 38B and 34C) and pyroptotic neutrophils (Figure 34C) were significantly reduced. This observation is interesting, as neutrophils are recruited to the injured lung after IRI. With a relatively short half-life, neutrophils undergo cell death and are replaced by macrophages in the infarct area. It has been shown that dead neutrophils can release ROS and granule components to cause secondary injury to the lung after an initial IRI. Thus, these results show that pyroptosis might be a key pathway of neutrophil death in lung tissue after IRI contributing to development of PGD and rhMG53 treatment could inhibit neutrophil pyroptosis and might preserve lung function.

***mg53*^{-/-} lung recipient mice show higher levels of pyroptosis and inflammation:**

Previous studies have established that MG53 repairs cell membrane injury and plays a critical role in protecting against lung injury. In addition, rhMG53 treatment reduced pyroptotic cells in IRI injury model (Figure 34). Next, the role of MG53 and pyroptosis was tested in a murine lung transplantation model. As shown in Figure 35, when WT lungs were transplanted in *mg53*^{-/-} recipient (WT-KO), significantly higher GsdmD-N was detected as compared to WT-WT transplantation lungs. The bronchioalveolar lavage (BAL) fluid collected in WT-KO mice also showed higher levels of inflammatory markers. These results show: (1) Pyroptosis happens in lung transplantation; (2) MG53 might play a role in controlling pyroptosis and inflammation associated lung transplantation IRI.

MG53 translocates to the plasma membrane but cannot inhibit activation of caspase-

1: To study the detailed function of MG53 during activation of pyroptosis, a human macrophage cell line, THP-1 cells (neutrophils are known to have a short lifetime of less than 10 hours in culture condition was used. Thus, THP-1 cells were used). First live cell imaging was performed with THP-1 cells overexpressing RFP-MG53. As it is shown in Figure 36A, activation of pyroptosis (treatment of LPS+Ni) led to rapid translocation of RFP-MG53 to the plasma membrane, showing

MG53 might translocate to plasma membrane injury site after induction of pyroptosis because application of rhMG53 reduces pyroptotic cells after IRI (Figure 34). Thus, the reduction of GsdmD cleavage by MG53 could be due to (1) inhibition of inflammasomes, which activate caspase-1 by proteolytically generating p20 and p10 fragments; (2) inhibition of caspase-1 mediated GsdmD cleavage. Here, THP-1 cells were treated using various concentrations of MG53 (0, 0.1, 1, 10 $\mu\text{g/ml}$) together with LPS+Ni (induction of pyroptosis). It was found that the activation of caspase-1, evaluated by the band intensity of p20 and p10, was not affected by MG53 (Figure 36B). Thus, MG53 might affect caspase-1 mediated GsdmD cleavage.

MG53 directly binds to GsdmD to inhibit its cleavage by caspase-1: To test the second possibility, a co-immunoprecipitation experiment was first performed. As shown in Figure 37A, MG53 and GsdmD could interact with each other. To test the potential function of MG53 on GsdmD cleavage by caspase-1, rhMG53 was incubated with caspase-1 before adding to GsdmD. As shown in Figure 37B (left), the inhibition of cleavage was prominent after 10 min, where a significant fraction of GsdmD was cleaved. The inhibition of the cleavage can be caused by direct inhibition of the enzyme caspase-1, or by an interaction of GsdmD and MG53 that protects the exposure of the cleavage site. Subsequently GsdmD fused with an N-terminal Sumo (small ubiquitin-related modifier) domain was used, which often increases the solubility of the fusion proteins using its surface hydrophilic residues. If MG53 interacts with caspase-1, the interaction should not be altered by the presence of Sumo domain on GsdmD. In that case, MG53 mediates inhibition of Sumo-GsdmD cleavage; on the other hand, if MG53 interacts with GsdmD instead of caspase-1, interaction between MG53 and GsdmD was observed to be reduced due to the addition of a Sumo domain. As shown in Figure 37B (right), cleavage of Sumo-GsdmD is entirely not affected by MG53, showing that MG53 inhibits GsdmD cleavage by binding to GsdmD, which is abolished by the presence of an N-terminal Sumo domain. Thus, it was concluded that MG53 binds to GsdmD N-terminus and inhibits caspase-1 mediated GsdmD cleavage.

MG53 suppresses the leakage of the liposome induced by GsdmD-N: While experiments in Figure 37 demonstrated that MG53 could inhibit caspase-1 induced GsdmD cleavage, it was still unclear whether MG53 could directly inhibit pyroptosis activity. A liposome reconstitution assay was performed as shown in previous publication, where GsdmD-N transmembrane pore formation can be quantified to serve as a functional indicator of pyroptosis activity. Briefly, liposomes containing fluorophore are treated with GsdmD and caspase-1. Caspase-1 could cleave and activate GsdmD forming transmembrane pores inducing the release of fluorescent dye inside the liposome. The released fluorophore is quenched by polyclonal antibodies

present in solution outside of liposome. Thus, by monitoring the dynamics of fluorophore quenching, the activation of pyroptosis can be quantified. When different doses of rhMG53 protein were added into liposome system, a clear reduction of pyroptosis activity was observed (with IC₅₀ of $9.1 \pm 4.2 \mu\text{g/mL}$) (Figure 38). Taken together, these data strongly support the role of MG53 in inhibiting pyroptosis.

Overall, the results disclosed herein show that MG53 antagonizes GsdmD-mediated pyroptosis. rhMG53 treatment following IRI suppressed pyroptosis (Figure 33) as compared to WT littermates. The focus of this study was to perform mechanistic experiments to reveal the mechanisms of action of MG53 in inhibition of pyroptosis in post-IRI injury. GsdmD was recently identified as the pyroptotic executioner upon enzymatic cleavage by inflammatory caspases-1/4/5/11. Upon cleavage, GsdmD-N dissociates from GsdmD-C and adopts an extended conformation to oligomerize and form pyroptotic pores. In addition to promoting cytokine release, the GsdmD pore disrupts osmotic potential, causing cell swelling and eventual rupture of the plasma membrane, a hallmark of pyroptosis. ESCRT machinery has been shown to repair GsdmD-induced membrane damage to resist pyroptosis. However, ESCRT blockage did not lead to 100% pyroptosis, showing there can be additional mechanisms for membrane repair after GsdmD activation. Given MG53 is a key component of cell membrane damage repair machinery in the lung, and it quickly accumulates on cell membrane upon pyroptosis (Figure 36), it was hypothesized that GsdmD-N formed plasma membrane pores can be repaired by MG53 and the data in Figure 37 show a direct binding between MG53 and cleaved GsdmD-N. Thus, it is determined how MG53 interacts with GsdmD to prevent its cleavage.

Experimental design:

Colocalization of MG53 and GsdmD-N upon pyroptosis: MG53 may interact with GsdmD-N (after pore formation). This is supported by the facts that 1. MG53 translocates to the plasma membrane after pyroptosis activation (Figure 36), 2. MG53 interacts with GsdmD (intact) but does not interact with sumoGsdmD (GsdmD-N might be blocked by sumo motif) (Figure 37), 3. Both GsdmD-N and MG53 have a high binding affinity to PS during cell membrane damage and repair. Time course of interaction between MG53 and GsdmD-N is determined by live cell imaging. Annexin V (a known PS binding protein) is used to show the involvement of PS in the interaction between GsdmD-N and MG53. Colocalization between MG53 and GsdmD-N is observed therefore, native gels are performed to investigate the possible physical interaction between MG53 & GsdmD-N after pyroptosis activation.

MG53 repairs membrane to inhibit pyroptosis. The ESCRT machinery and MG53 are two parallel systems that sense and repair the damaged membrane using a similar mechanism. ESCRT repairs GsdmD-N induced membrane injury, but deletion of ESCRT does not lead to 100% cell pyroptosis, showing there can be an alternative pathway for membrane repair after GsdmD activation.

Results:

Results of Colocalization of MG53 and GsdmD-N upon pyroptosis: It has been shown in the data and previous publications that both MG53 and GsdmD-N can be labeled in lung tissue. As a result, the two proteins colocalize after pyroptosis, showing they directly or indirectly interact as part of a protein complex.

The effective dose and time window for rhMG53 mediated protection against pyroptotic death.

In a sub-acute mouse transplant 5-day survival model (Figure 39) of genetically ablated and over-expressed MG53, changes in the pyroptotic pathway and GsdmD cleavage are demonstrated. Through left lung transplantation after 1 hour of warm ischemic injury (that models a donation after circulatory death IRI injury) WT donor into a WT or genetic ablation of MG53 (*mg53*^{-/-}) recipients, the *mg53*^{-/-} recipients showed significantly higher inflammatory markers in the bronchioalveolar lavage (BAL) fluid (Figure 41).

Based on these findings, it was hypothesized that while initial neutrophil infiltration plays a reparative role to lung donor allografts after IRI, infiltrated neutrophils undergo pyroptosis in a harsh injured environment that causes secondary injury to the pulmonary parenchyma. Thus, if neutrophil pyroptosis can be prevented, the beneficial function of neutrophils is maximized for pulmonary repair and remodeling.

Neutrophils from *mg53*^{-/-}, tPA-*mg53*, *GsdmD*^{-/-}, and WT littermate mice are isolated freshly. Dose and time effects of rhMG53 are investigated upon induction of pyroptosis in neutrophils (Figure 39). The protocol for induction of neutrophil pyroptosis is: 1-hour LPS priming, followed by administration of Ni. To study the temporal role of rhMG53, three different timings are used (Figure 36), 10 min before LPS priming, 10 min before adding Ni, and 10 min after adding Ni. Priming is the first signal and critical for inflammasome assembly and thus important for caspase 1 activation and GsdmD cleavage. At each time point, different doses of rhMG53 (0, 0.1, 1, 10, 100 µg/ml) are used to determine an effective dose of rhMG53 in protecting neutrophils against pyroptosis. Live cell imaging of FM1-43 dye entry into cells is used as an index for cell membrane injury. In addition, pyroptotic markers, GsdmD-N, and caspase-1

activation are assessed using WB as endpoint measurement. The release of LDH into the culture medium in response to various experimental manipulations are used as an alternative method to assess cell membrane integrity.

The impact of MG53 is investigated on time course of inflammation and pyroptosis evolution. Single cell RNA-seq analysis of lung is conducted (n=3/grp) after hilar clamp IRI (Figure 40) in WT, tPA, and *mg53*^{-/-} mice and have found a 2-fold increase neutrophil expression of caspase-1 in *mg53*^{-/-}, as compared to WT and a near 0 increase in the tPA cohort, showing the MG53-mediated regulation of pyroptosis in neutrophils. By co-culture models of IRI (Figure 32) the time and impact of MG53 in modulating pyroptosis and expand to precision cut lung specimens for tissue level assessments are explored results: 1, general effects of rhMG53 on pyroptosis: As shown in the data (Figures 34, 37), it was found that MG53 reduced pyroptosis post-IRI, during which neutrophils are the dominant cells undergoing pyroptosis. A reduction of GsdmD cleavage is observed by adding rhMG53 to WT and *mg53*^{-/-} neutrophils. *GsdmD*^{-/-} cells are a negative control for neutrophil pyroptosis and membrane damage; tPA-MG53 neutrophils and overexpressing endogenous MG53 are protected from pyroptosis without rhMG53. 2, dose effects of rhMG53: Dose-effects of rhMG53 on WT neutrophils elucidate whether MG53 could fully block pyroptosis at high concentrations. The data of MG53's effects on liposome leakage (Figure 38) showed an incomplete blockage of leakage. Either complete or partial blockage highlights a new mechanism of MG53 to protect lung IRI.

Timing effects of rhMG53: Protective effects are observed when rhMG53 is added before adding Ni or LPS. When MG53 repairs pyroptosis induced membrane damage, significant protection with MG53 added 10 min post Ni is observed.

The role of MG53 and neutrophils in pulmonary IRI pyroptotic damage in mice models: There are many cell types in the pulmonary parenchyma, e.g., alveolar macrophages, interstitial macrophages, AT1 and AT2 pneumocytes, neutrophils, fibroblasts, and endothelial cells. The ischemic insult could lead to cell death of all types of cells in the lung, with the release of many cellular factors with varying biological roles. Although it was found that GsdmD-N is primarily located in neutrophils after IRI (Figure 34), it does not rule out the possibility that other cell types do not undergo pyroptosis or contribute to pulmonary damage. As disclosed herein, is a lung atlas nomenclature and found a 90-min hilar clamp leads large changes in pyroptotic pathway signaling and is most significant in leukocyte, specifically neutrophil populations (Figure 40). At 90-min IRI, there appears minimal apoptotic pathway signaling which is in stark contrast to the apoptotic signal seen in the lung at 5-days post transplantation (Figure 39D).

IRI Secondary to Lung Hilar Clamp: A left lung hilar clamp model of 90-min occlusion is used to induce IRI in the lung of C57BL/6 mice, *GsdmD*^{-/-}, *mg53*^{-/-} and tPA-MG53 mice (n=8/group). Groups consist of: (1) sham injury, (2) IRI + vehicle, (3) IRI + dose escalation of rhMG53, (4) IRI + disulfiram (0-40 μ M), and (5) IRI + rhMG53 + disulfiram. One cohort of mice is used to perform histological, biochemical, and biomolecular analysis with tissue. For histology, a portion of the lung is processed for H&E and lung injury scores are assessed. BAL is collected to look for protein, total cells, and neutrophils. Lung tissue is collected for WB (IL-1, IL-6, TNF- α , ET-1), wet/dry ratio, MPO activity, and qPCR for inflammation. Pulmonary function is assessed by plethysmography and Flexivent prior to sacrifice of the survivors. In another cohort of mice, raw survival from injury is assessed via the Kaplan-Meier method, and body weight is monitored with daily plethysmography. BAL and serum are collected at the time of sacrifice. The right upper lobe is collected for histology and immunohistochemistry.

Measurement Methods and Endpoints: Efficacy is assessed by physiologic and molecular metrics established in PIs' labs (Figures. 33-34). Biometric signature of molecular markers is employed (ET-1, BigET- 1, LDH, HA, IL-1 β , NLRP-3, caspase-1/3/7, flow cytometry) and pulmonary function (*Flexivent and plethysmography*) and arterial blood oxygenation (ABG). Lung injury is assessed by lung permeability index (the ratio of BAL) protein to serum protein concentrations. Wet-to-dry ratio is evaluated by the severity of pulmonary edema and lung tissue is used to create lysate to measure biomarkers. Published studies have shown that ET-1 expression is controlled by numerous stressors on endothelial cells (e.g., cytokine, oxidative stress, hypoxia shear) and increased ET-1 levels are modulated by hypoxia or cytokine induction of endothelin converting enzyme (ECE) activity. The role of ET-1 as a marker of lung injury or rejection post-transplant has been well established. Endothelial cell IRI induces generation of ROS which may contribute to elevation of ET-1.

IRI Secondary to murine Lung Transplantation with Warm Ischemia: To assess these interactions and the putative therapeutic benefit of rhMG53, lung transplantation is performed after warm ischemia to induce a significant (yet not fatal) IRI analogous to a donation after circulatory death human transplant. Biometric signature of lung injury with increasing warm ischemic times of 0-, 30-, 60-, 90-, 120-min warm ischemia was utilized and have identified a 60-min warm injury as substantial yet not catastrophic. This mimics the human experience. It has been well established that this isolated left lung transplant model with the genetically modified mouse lines and survival out to 120-days in WT control (Figure 41). It was found that when *mg53*^{-/-} mice were utilized as recipients, there are significantly higher inflammatory responses as evidenced by BAL cell count

(Figure 41D), BAL protein (Figure 41E), and LDH in BAL (Figure 41F). With this established animal model, this study focuses on elucidating the *in vivo* function of MG53 on modulating neutrophil pyroptosis for lung protection. The recipient mice are *GsdmD*^{-/-}, because the first focus is on determining the function of pyroptosis in the infiltration into the donor allografts.

5 As *GsdmD* expresses and pyroptosis might happen in pulmonary tissues with systemic effects of transplant IRI, *GsdmD*^{-/-} mice are ideal as a recipient mouse line to eliminate interference caused by pyroptosis from whole animal. The following series of transplantation experiments are used to specifically assess the role of neutrophils in transplant IRI. All mice of listed genotypes are available for the protocol. Completion of the studies have led to show that 1, the extent that
10 neutrophils contribute to pyroptosis-induced post-IRI damage, 2, if MG53 directly inhibits neutrophil pyroptosis *in vivo*, 3, if the cell type origin of MG53 to inhibit post-IRI pyroptosis. Briefly, left donor lung allografts with 60-min warm ischemia are transplanted *GsdmD*^{-/-} mice. A group of mice are sacrificed 24 hours after surgery, with lungs, serum, and BAL being collected. In a second set, recipient mice survive a week from transplantation with serum collection at 1-, 3-,
15 7-days. Standard histology, ELISA and immunohistochemistry are employed. Longitudinal pulmonary function assessed via plethysmography at 1-, 3-, 7-days post-transplant (Plethysmography enables non-invasive, longitudinal pulmonary function, and lung mechanics data over the post IRI period. This enables the study to conserve animals and minimize stress due to sedation) and Flexivent is conducted prior to sacrifice. Transplanted lungs contain cell types
20 other than neutrophils, such as macrophages that might contribute to IRI. For this purpose, it has been successfully generated neutrophil specific *GsdmD*^{-/-} mice by breeding *LysMCre* mice with *GsdmD* *fl/fl* mice. As the time of writing, six *LysMCre*^{+/+}:*GsdmD* *fl/fl* mice were present in the mouse colonies. The mice show similar phenotypes as their littermate controls, showing conditional *GsdmD*^{-/-} does not interference with mouse physiology in basal condition. This
25 interrogates the neutrophil contribution.

Large animal and human lung EVLP with rhMG53 to preserve and rescue lung allograft integrity.

A study was conducted with the porcine model of EVLP after 1-hour warm ischemia and found that treatment with rhMG53 via EVLP could preserve lung parenchymal architecture with
30 minimal interstitial edema (Figure 42), mitigating the inflammatory cytokine response (IL-6) measured in the perfusate and a preservation of overall lung architecture. Additionally, in the prior funding period, It has been demonstrated that a therapeutic benefit of rhMG53 administration to

protect lung IRI after a prolonged cold static preservation period (Figure 43) with marked improvement in recipient oxygenation and ventilation.

This study allows to study a) the use of porcine model of EVLP and lung transplantation to test whether the addition of rhMG53 the preservation process or after transplantation can have beneficial effects to prevent allograft injury; and b) test human lung tissue to support the premise of rhMG53 supplementation in transplant relevant IRI.

Therapeutic window of rhMG53 administration for organ preservation and EVLP assessment followed by transplantation: Based on these data, the following set of porcine studies

as outlined in Figure 59 are performed. The proposed experimental groups are designed to test the injury associated with warm ischemia, the cold ischemia during storage and transport, and the injury associated with normo-thermic EVLP. First, rhMG53 is added prior to cardiac arrest in the donor animal to determine the preventive effect of rhMG53 against ischemic injuries (Point A). Second, rhMG53 can then be added to the normothermic EVLP Steen[®] solution (Point C), to assay the resuscitative effect of MG53 in preservation of to the lung during the EVLP reconditioning procedure or prior to reperfusion (Point D). In these cohorts, isolated left single lung transplant are performed in the heparinized recipient. The explanted donor lung is used for assessment of endothelial cell health and lung integrity. At the termination of the recipient transplant reperfusion period (6-hour), the native right and transplanted left lung is recovered and analyzed. Standard clinical endpoints assess allograft integrity prior to lung transplantation and tissue and perfusate are banked for post-hoc analysis. At the termination of the recipient transplant reperfusion period, the native and transplanted lung is recovered and evaluated. Immediately prior to euthanasia, the right pulmonary artery is occluded and a 100% ventilated FiO₂ is used with assessment of pulmonary mechanics to calculate terminal PaO₂/FiO₂ (P/F) ratio and function. Standard porcine immunosuppression is administered. Power calculations based on P/F ratio and LDH data requires 6 pigs/group.

rhMG53 delivered to poor-quality human donor allografts as a rescue agent: It was found that in the poor-quality lung not able to be rescued, there is a marked increase in apoptotic injury, as assessed by TUNEL/DAPI staining (Figure 44). Through a biorepository and the human lung transplant program at OSU, the study has access to the unused donor lungs (~20 per year). These donors according to the traditional criteria are non-transplantable. EVLP is performed for 4 hours.

Example 5: Mitsugumin-53 Preservation of Lung Function after prolonged ischemia and transplantation.

As the donor availability is expanded, elongated ischemic periods occur resulting in increased ischemia reperfusion injury (IRI) which can lead to quality issues, and development of primary graft dysfunction (PGD). Prevention and treatment of IRI would be a therapeutic approach to rescue potential donor allografts and mitigate PGD. Mitsugumin-53 (MG53) is part of endogenous cell membrane repair machinery, and exogenous administration of MG53 can mitigate IRI. In this study, it was aimed to identify a therapeutic benefit of MG53 to preserve allograft function, primarily at the endothelial cell level, to improve transplantation outcomes.

Methods:

Yorkshire pigs were randomly assigned into two groups, exogenous MG53 or vehicle administration (n=6) and underwent left single lung transplantation after the donor allograft was subjected to 24h of cold static storage (CSS). Following reperfusion, the recipient pigs survived for 4h. Baseline blood samples of all donors were collected, as well as tissue samples of the donor allograft after 24h of CSS. During the transplant procedure, systemic arterial blood samples were taken throughout at set intervals for analysis. At sacrifice, isolated blood gases were taken from the donor and native pulmonary veins (PV). Additionally, blood samples, tissue and bronchoalveolar lavage (BAL) samples from both donor and native lungs were collected at sacrifice. Blood, tissue and BAL were analyzed for biochemical and histological markers of lung injury.

Results:

Median systemic PaO₂ at the end of the reperfusion period for the MG53 group was 357.0 (107.0-489.0) as compared to 152.8 (54.2-470.0) for the vehicle control (p=0.18). There was a significantly higher PaO₂ in the isolated PV of the MG53 group (465.0; 354.0-489.0) compared to the vehicle group (102.7; 58.5-489.0) (p<0.05) (Figure 58A). Compared to their baseline systemic PaO₂, pigs treated with vehicle control had significantly lower final systemic PaO₂ (p<0.05), while this relationship did not hold true with the MG53 treated group. Median systemic PaCO₂ at the end of the reperfusion period was significantly lower in the MG53 treated group (40.95; 38.0-49.5) compared to the vehicle group (55.15; 41.4-91.1) (p<0.05) (Figure 58B). Additionally, the PaCO₂ from both the isolated PV of the native and transplanted lung were significantly lower in the MG53 treated group (p<0.05). Median systemic pH was significantly lower in the vehicle treated group (7.351; 7.149-7.498) compared to MG53 treated group (7.503; 7.408-7.534) at the end of the

reperfusion period ($p < 0.05$) (Figure 58C). Sodium was significantly elevated in the vehicle treated transplanted isolated PV as compared to the MG53 treated group ($p < 0.05$) at the end of the reperfusion period. Plasma analysis during reperfusion showed a significant elevation of lung injury marker lactate dehydrogenase (LDH) in the vehicle treated group at 30-minutes and 1h post-reperfusion compared to the MG53 treated group ($p < 0.05$) (Figure 58D).

Conclusion:

In this porcine model of transplantation, exogenous MG53 administration had the ability to rescue injured allografts after prolonged CSS, prevent injury and return them close to a physiologic baseline. This study builds on the premise that exogenous MG53 administration functions as a therapeutic during lung transplantation mitigating IRI.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments of the invention and that such changes and modifications can be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

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SEQUENCES

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PEGAEA

CLAIMS

What is claimed is:

1. A perfusate composition comprising recombinant human Mitsugumin-53 (rhMG53) protein and a perfusion medium.
2. The perfusate composition of claim 1, wherein the rhMG53 comprises SEQ ID NO: 1.
3. A preservation solution comprising recombinant human Mitsugumin-53 (rhMG53) protein and a preservation medium.
4. The preservation solution of claim 3, wherein the rhMG53 comprises SEQ ID NO: 1.
5. A method of treating or preventing ischemia reperfusion injury (IRI) in an *ex vivo* graft comprising administering a recombinant human Mitsugumin-53 (rhMG53) protein to the *ex vivo* graft.
6. The method of claim 5, wherein the rhMG53 protein is administered in a perfusion medium.
7. The method of claim 5, wherein the rhMG53 protein is administered in a preservation medium.
8. The method of any one of claims 5-7, wherein the rhMG53 comprises SEQ ID NO: 1.
9. The method of any one of claims 5-8, wherein the rhMG53 protein is administered to the *ex vivo* graft before reperfusion of the *ex vivo* graft.
10. The method of any one of claims 5-9, wherein the rhMG53 protein is administered to the *ex vivo* graft during reperfusion of the *ex vivo* graft.
11. The method of any one of claims 5-10, the rhMG53 protein is administered to the *ex vivo* graft for at least 1 hour.
12. The method of any one of claims 5-11, wherein the *ex vivo* graft is an allograft or a xenograft.

13. The method of any one of claims 5-12, wherein the *ex vivo* graft is a lung, liver, heart, kidney, or intestine graft.
14. A method of treating a subject with primary graft dysfunction (PGD) comprising administering a recombinant human Mitsugumin-53 (rhMG53) protein to the subject.
15. The method of claim 14, wherein the rhMG53 protein is administered in a perfusion medium.
16. The method of any one of claims 14-15, wherein the rhMG53 comprises SEQ ID NO: 1.
17. The method of any one of claims 14-16, wherein the rhMG53 protein is administered intravenously.
18. The method of any one of claims 14-17, wherein the rhMG53 protein is administered subcutaneously.
19. The method of any one of claims 14-18, wherein the rhMG53 protein is administered before transplantation of an *ex vivo* graft.
20. The method of any one of claims 14-19, wherein the rhMG53 protein is administered during transplantation of an *ex vivo* graft.
21. The method of any one of claims 14-20, wherein the rhMG53 protein is administered after transplantation of an *ex vivo* graft.
22. The method of any one of claims 19-21, wherein the *ex vivo* graft is an allograft or a xenograft.
23. The method of any one of claims 19-22, wherein the *ex vivo* graft is a lung, liver, heart, kidney, or intestine.
24. The method of any one of claims 14-23, wherein the subject is human.

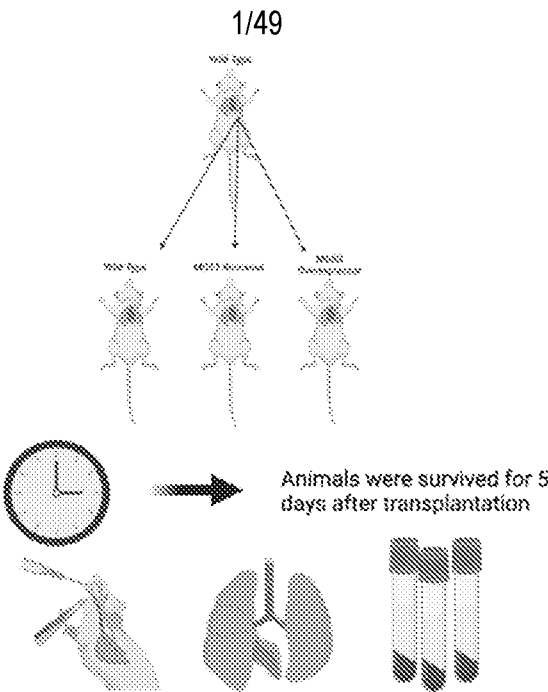


FIG. 1A

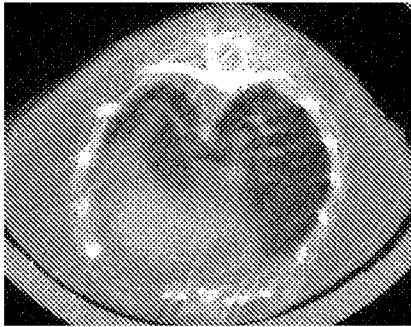


FIG. 1B

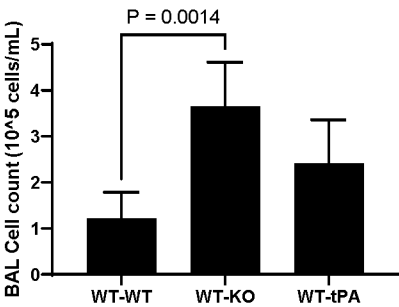


FIG. 2A

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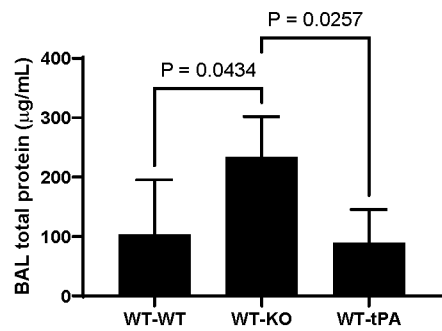


FIG. 2B

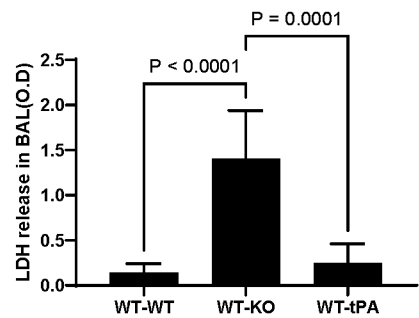


FIG. 2C

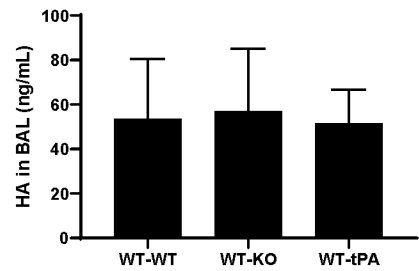
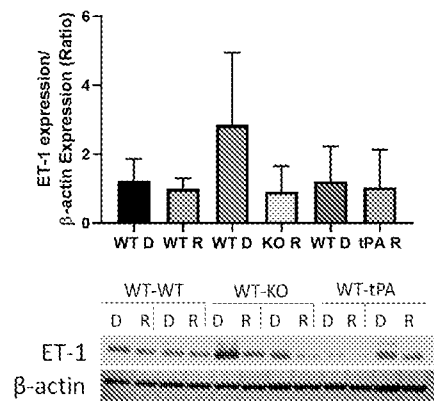


FIG. 2D



WT: WT mouse
KO: MG53 knock out mouse
tPA: MG53 overexpression mouse
D: Donor's Transplanted lobe
R: Recipient's lobe post transplantation

FIG. 3A

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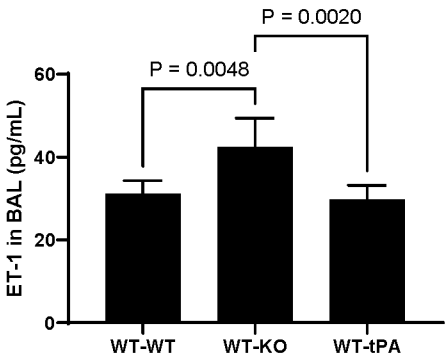


FIG. 3B

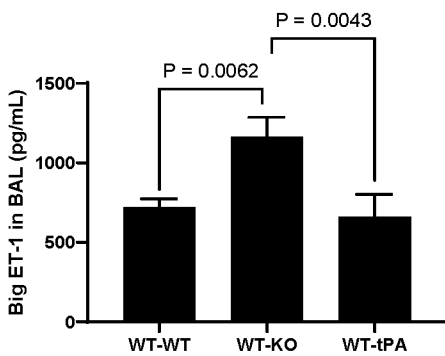
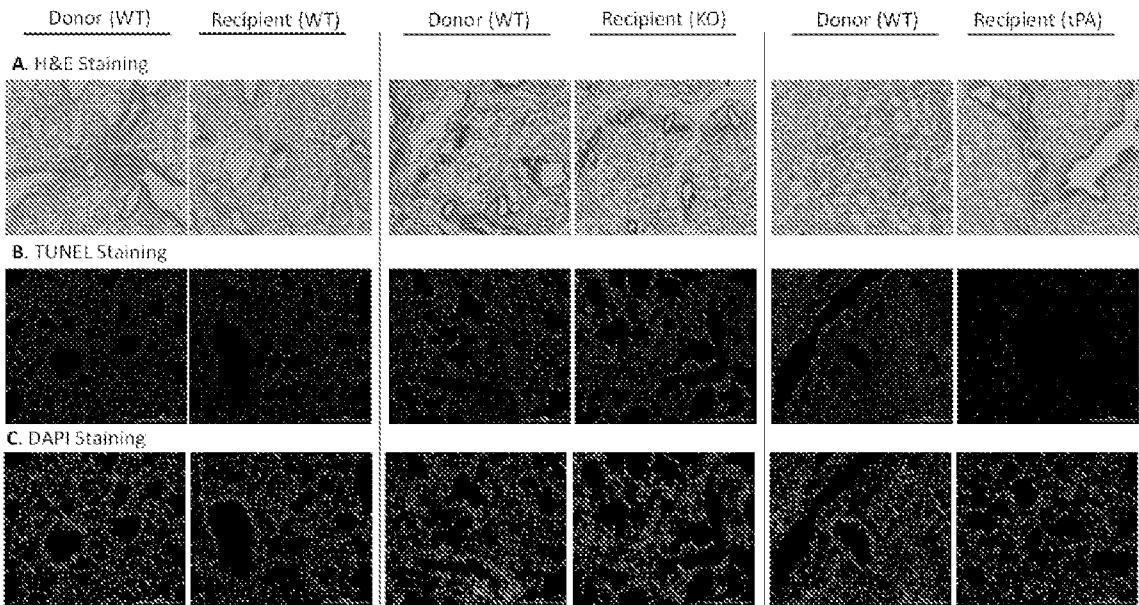


FIG. 3C



FIGS. 4A, 4B, 4C

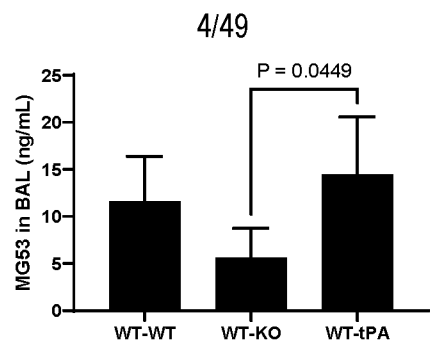


FIG. 5A

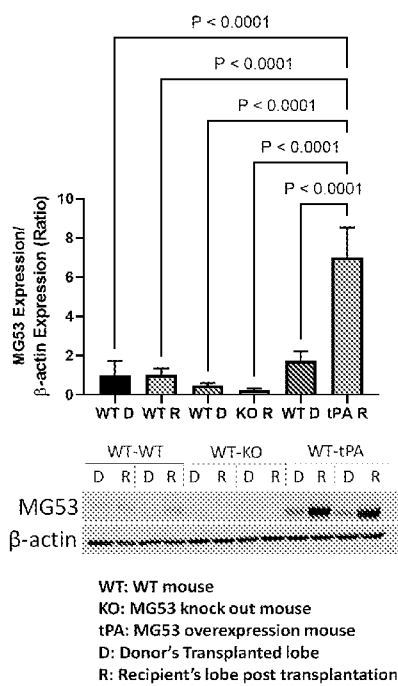


FIG 5B

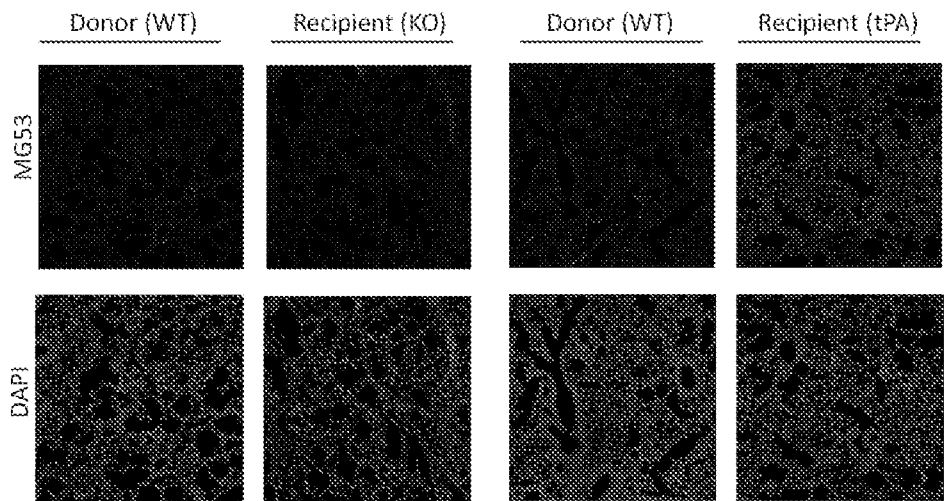


FIG. 6A

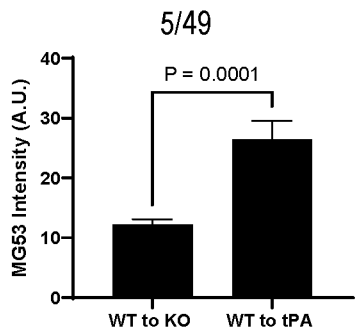


FIG. 6B

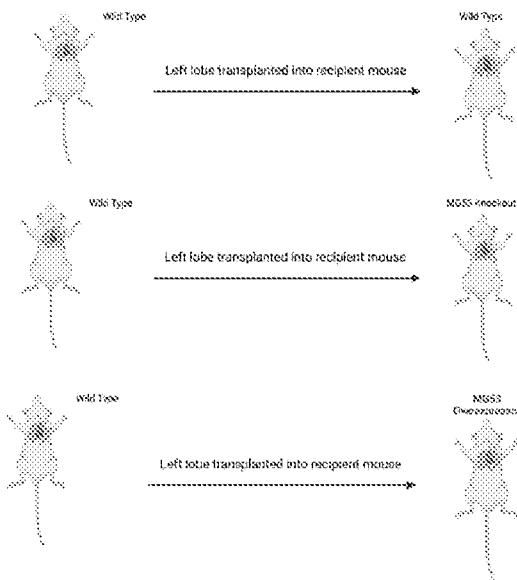


FIG. 7

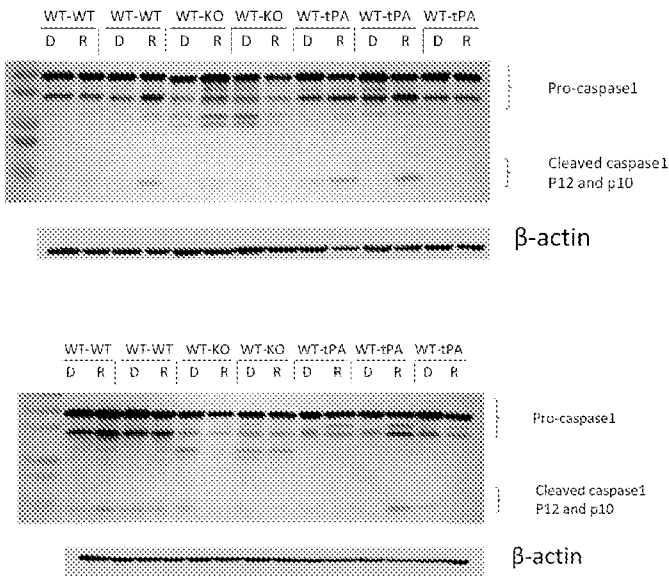


FIG. 8A

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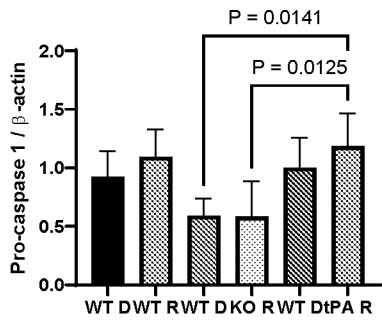


FIG. 8B

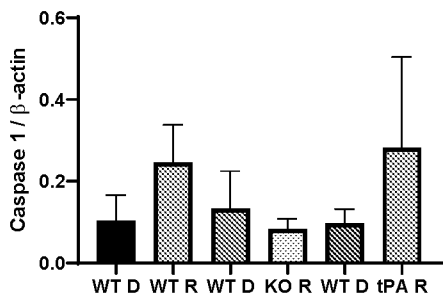


FIG. 8C

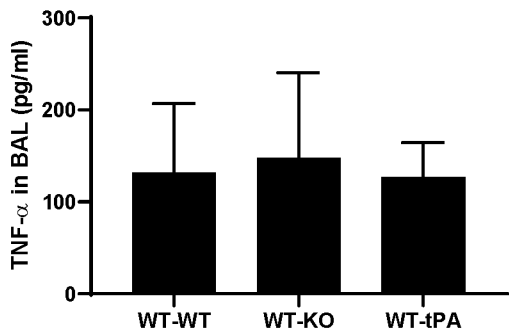


FIG. 9A

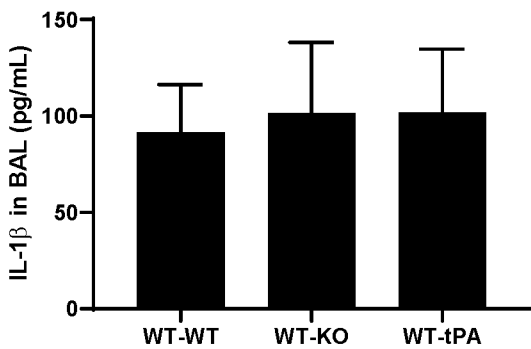


FIG. 9B

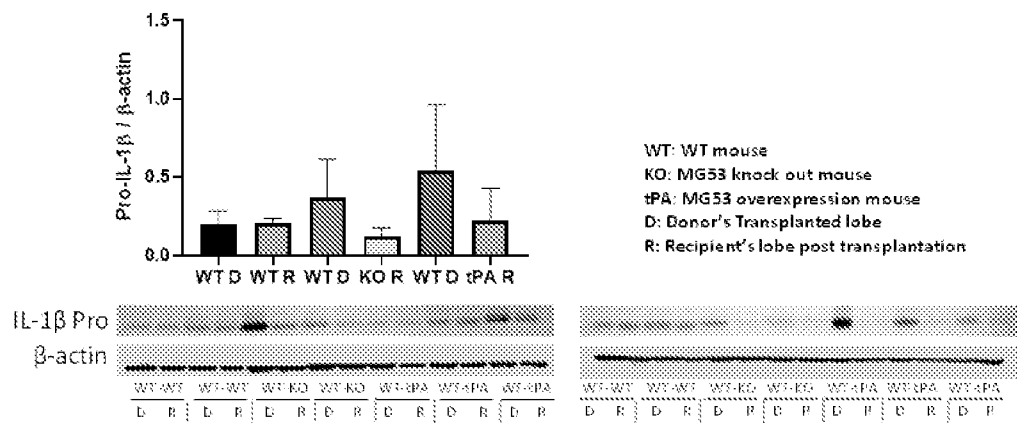


FIG. 9C

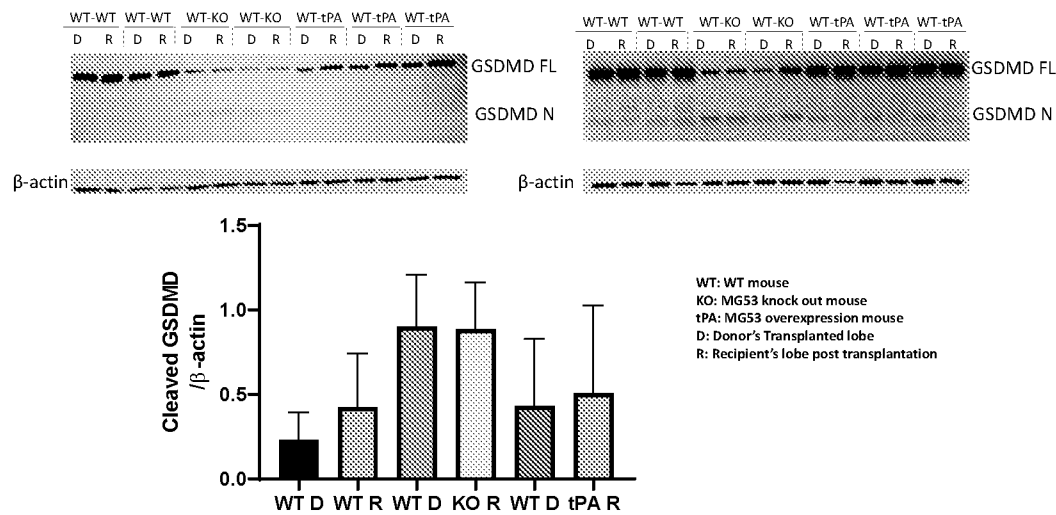


FIG. 10

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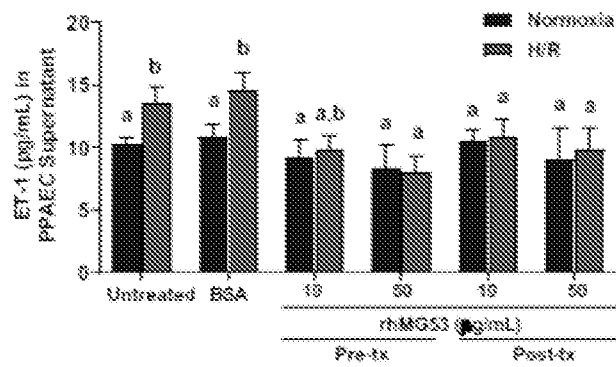


FIG. 11A

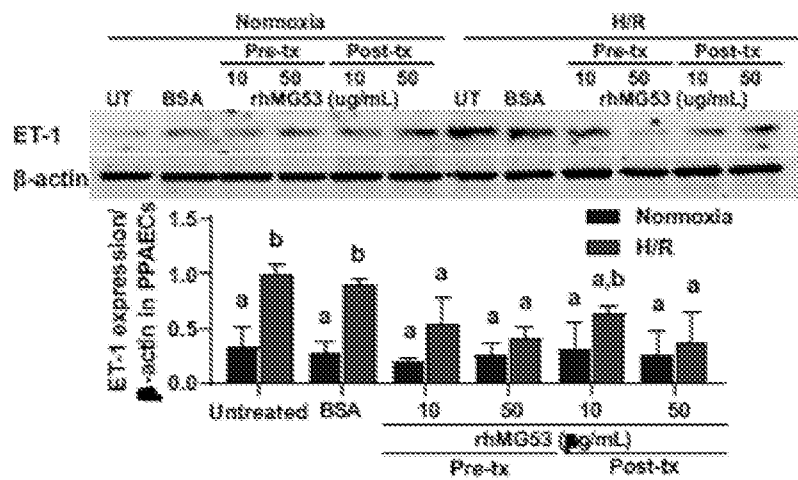


FIG. 11B

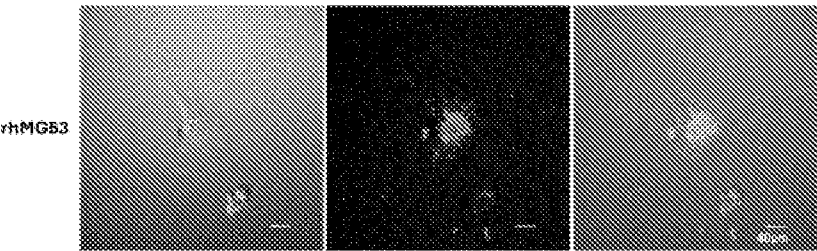


FIG. 11C

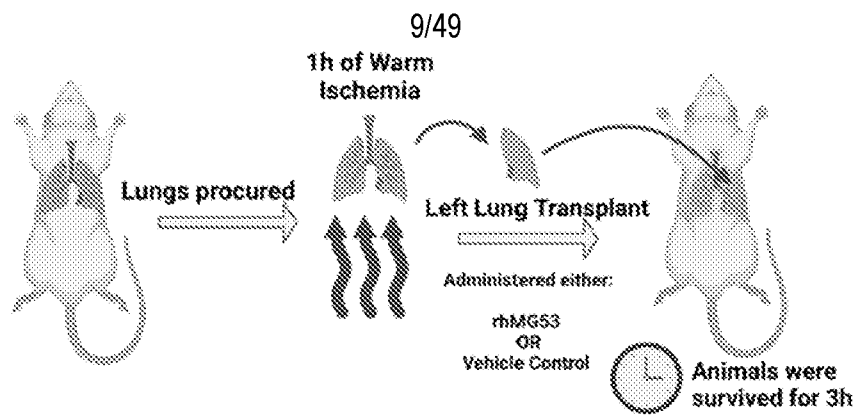


FIG. 12A

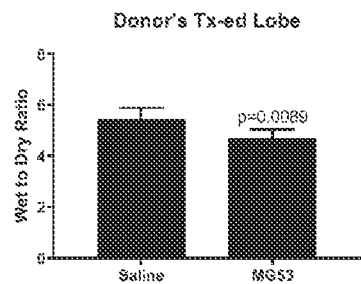


FIG. 12B

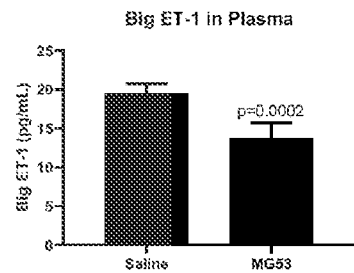


FIG. 12C

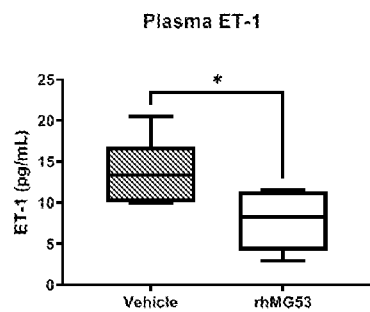


FIG. 12D

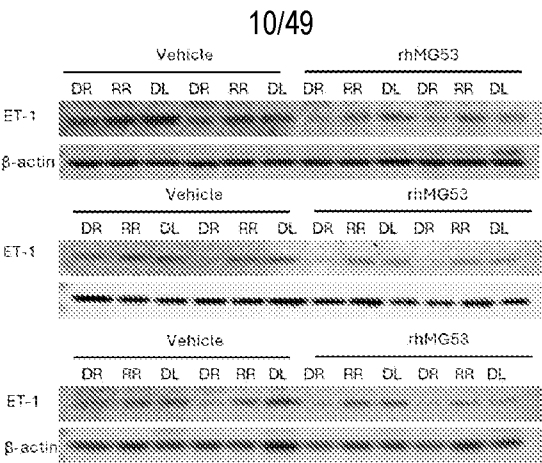


FIG. 12E

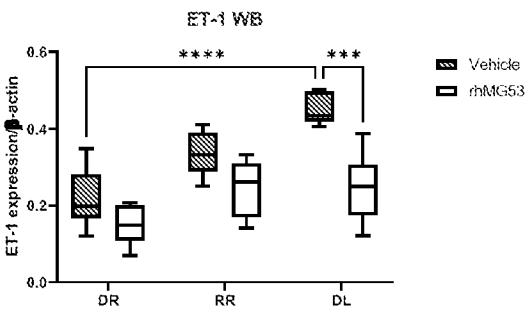


FIG. 12F

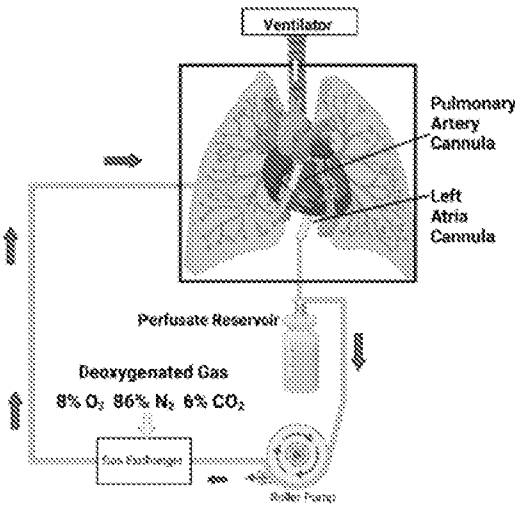


FIG. 13A

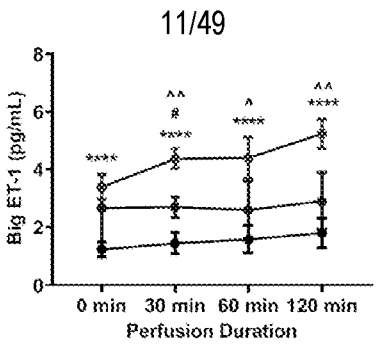


FIG. 13B

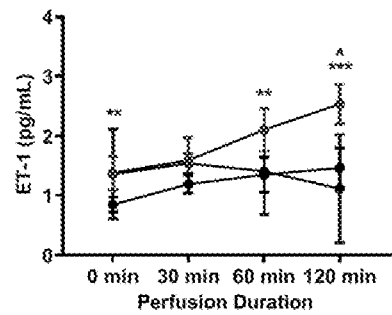


FIG. 13C

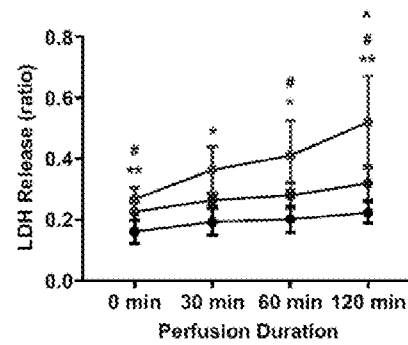
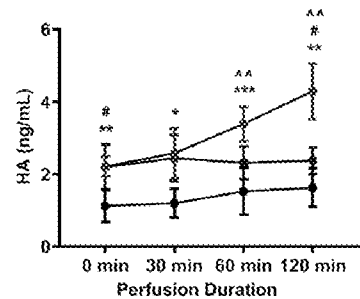


FIG. 13D



● 0h WI
● 1h WI
● 1h WI + rhMG53

FIG. 13E

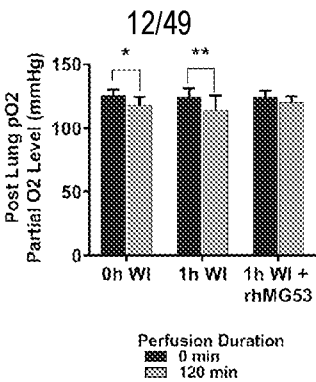


FIG. 13F

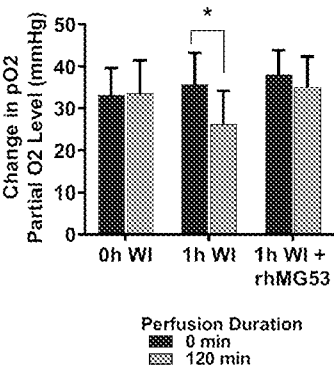


FIG. 13G

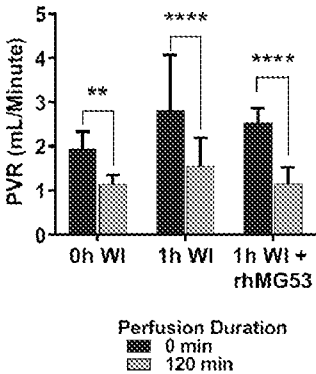


FIG. 13H

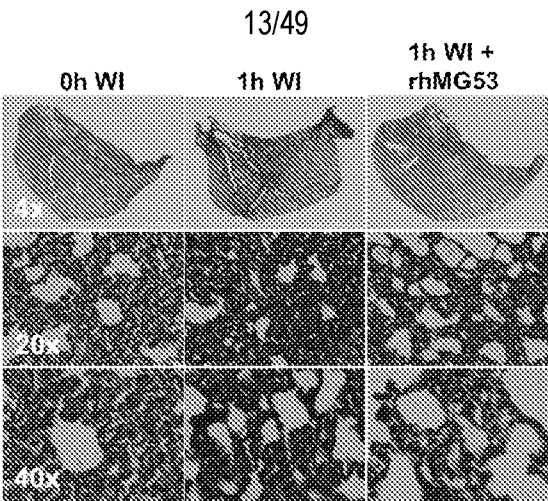


FIG. 14A

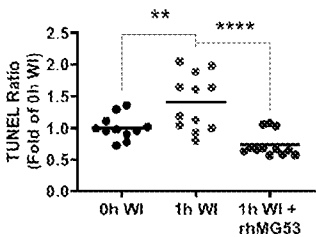
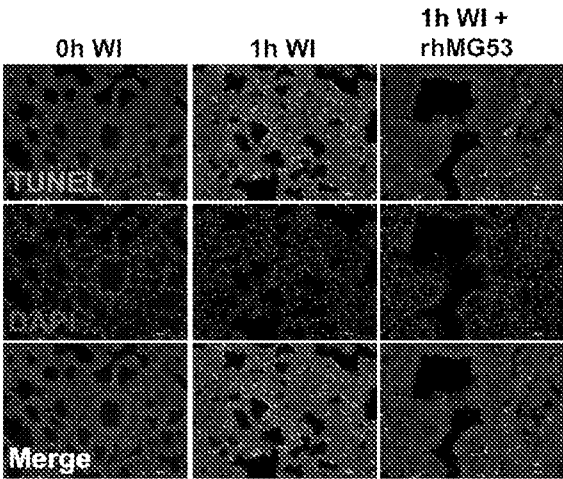


FIG. 14B

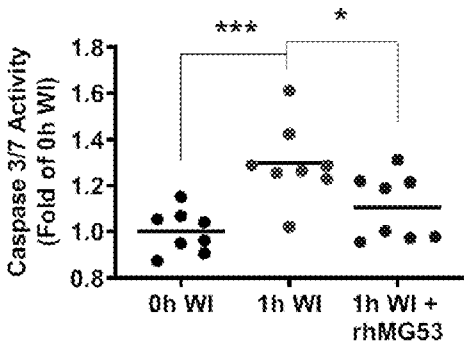


FIG. 14C

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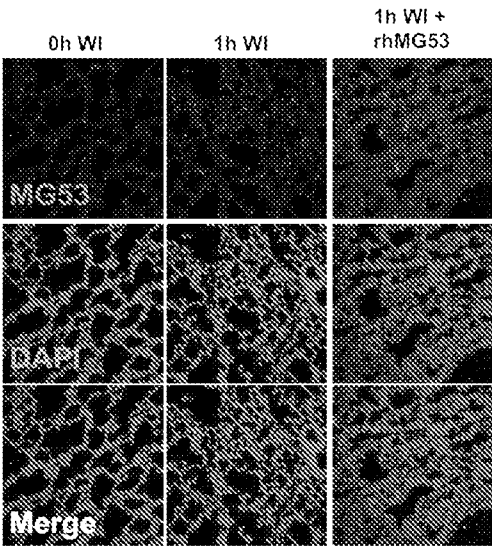


FIG. 15A

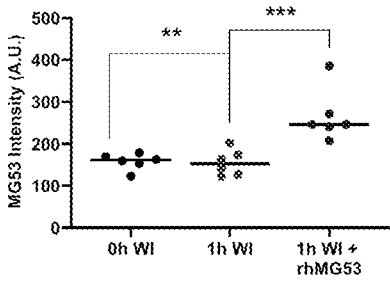


FIG. 15B

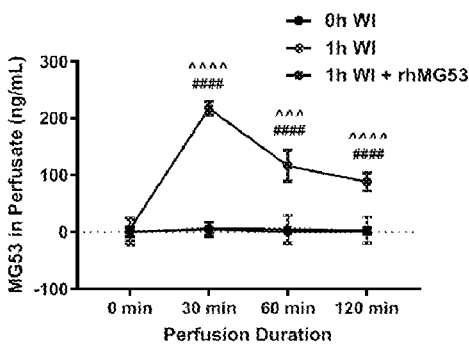


FIG. 15C

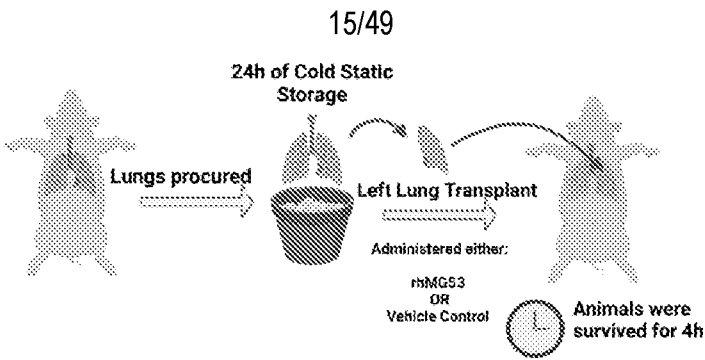


FIG. 16A



FIG. 16B

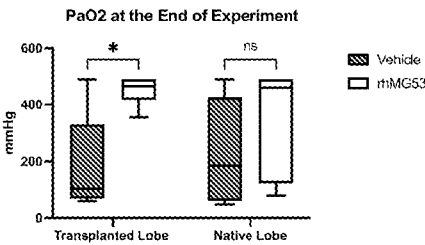


FIG. 16C

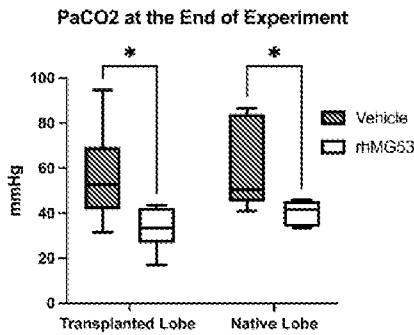


FIG. 16D

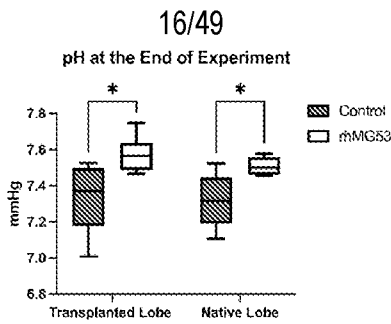


FIG. 16E

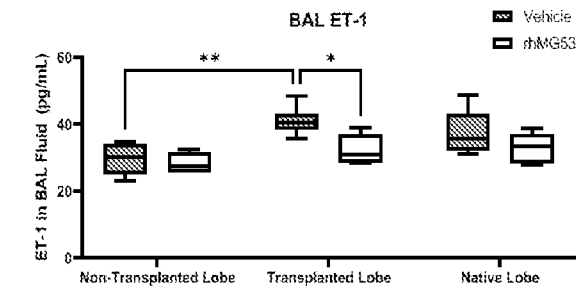


FIG. 16F

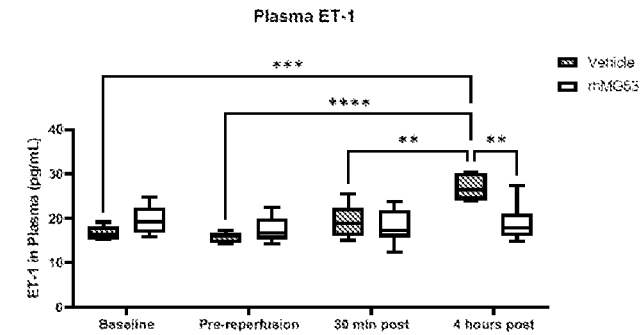


FIG. 16G

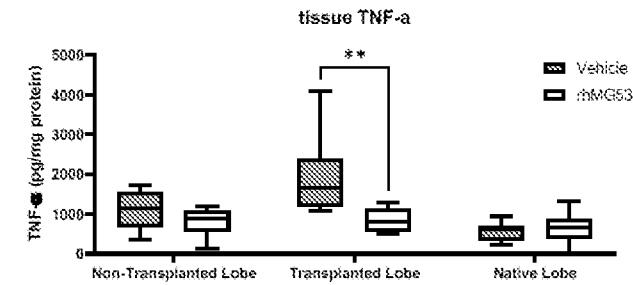


FIG. 17A

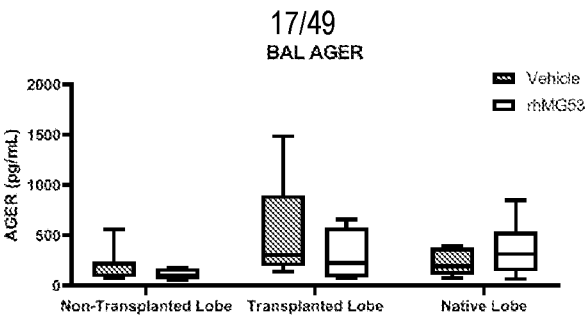


FIG. 17B

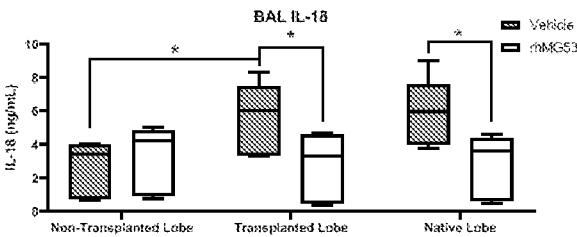


FIG. 17C

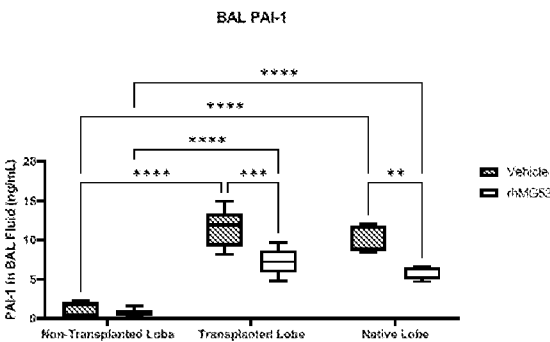


FIG. 17D

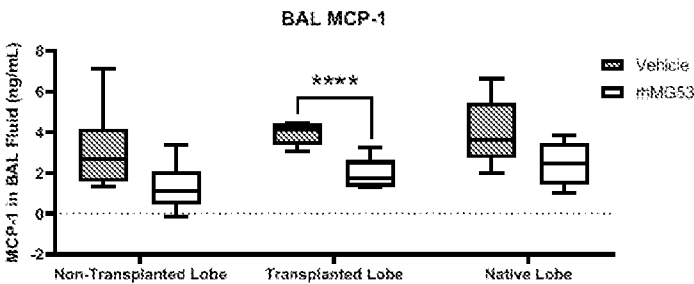


FIG. 17E

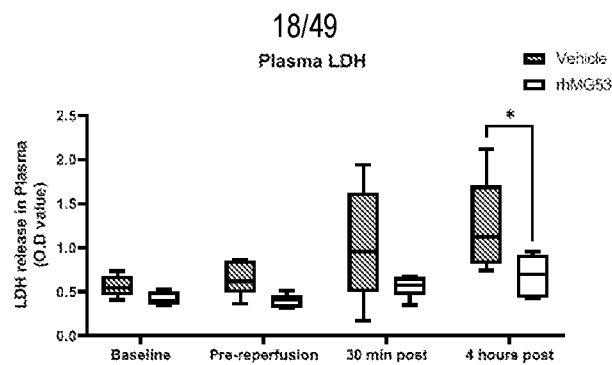


FIG. 17F

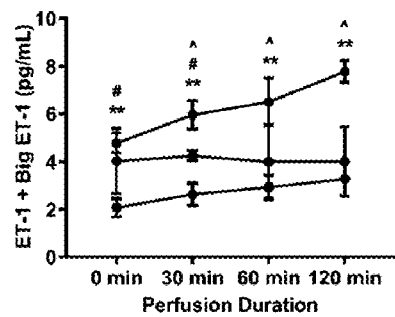


FIG. 18A

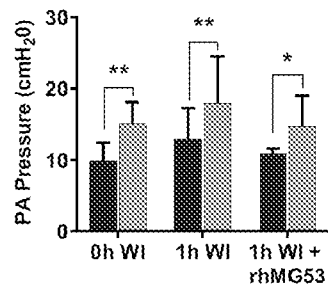


FIG. 18B

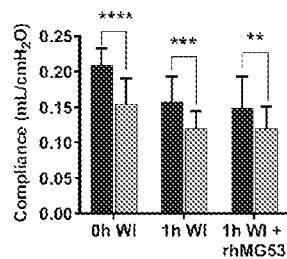


FIG. 18C

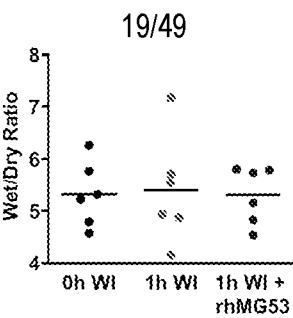


FIG. 18D

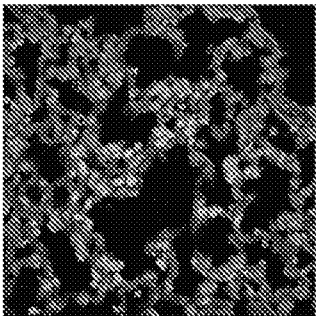


FIG. 19

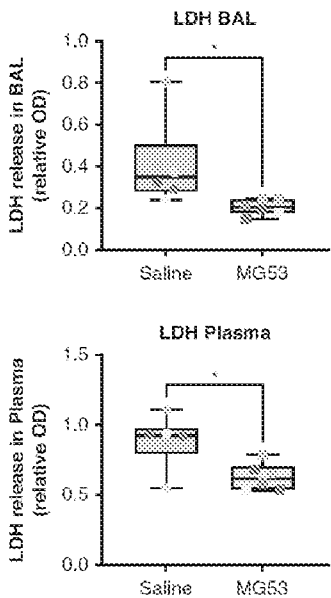


Fig. 20A

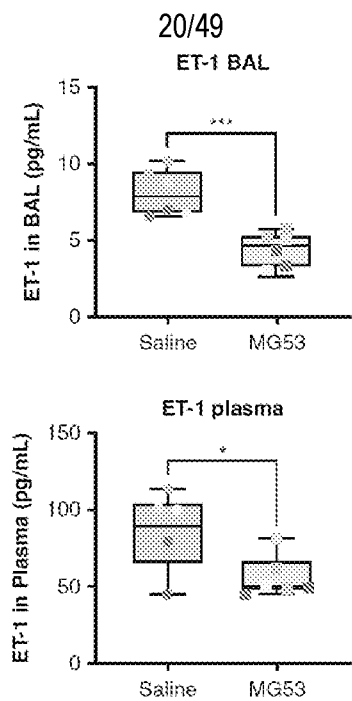


FIG. 20B

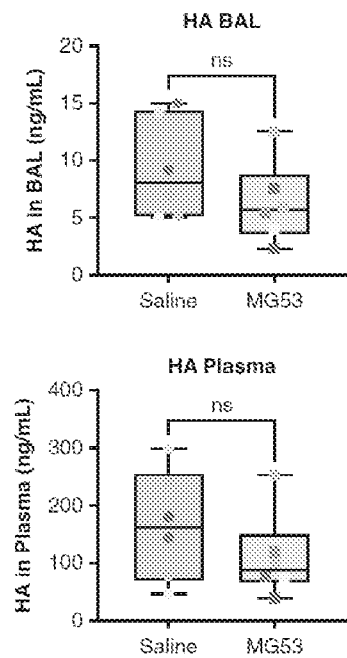


FIG. 20C

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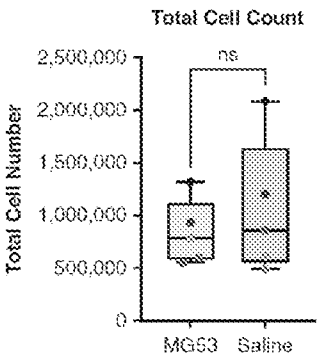


FIG. 21A

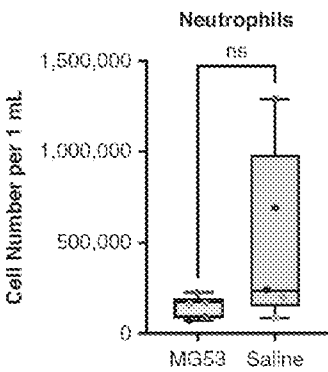


FIG. 21B

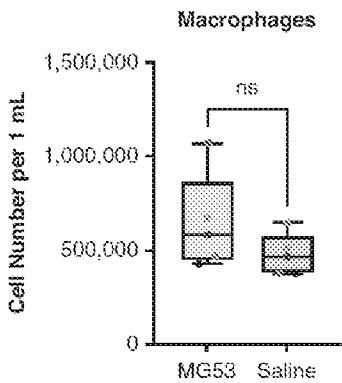


FIG. 21C

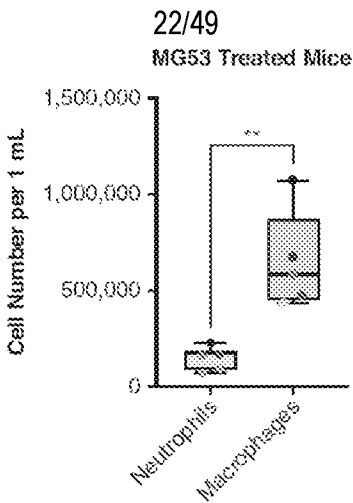


FIG. 21D

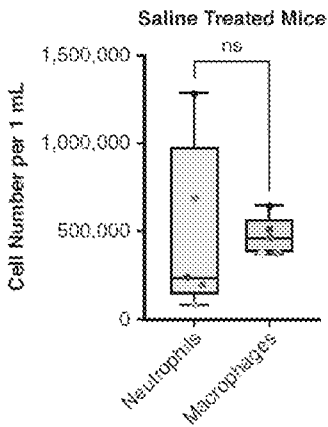


FIG. 21E

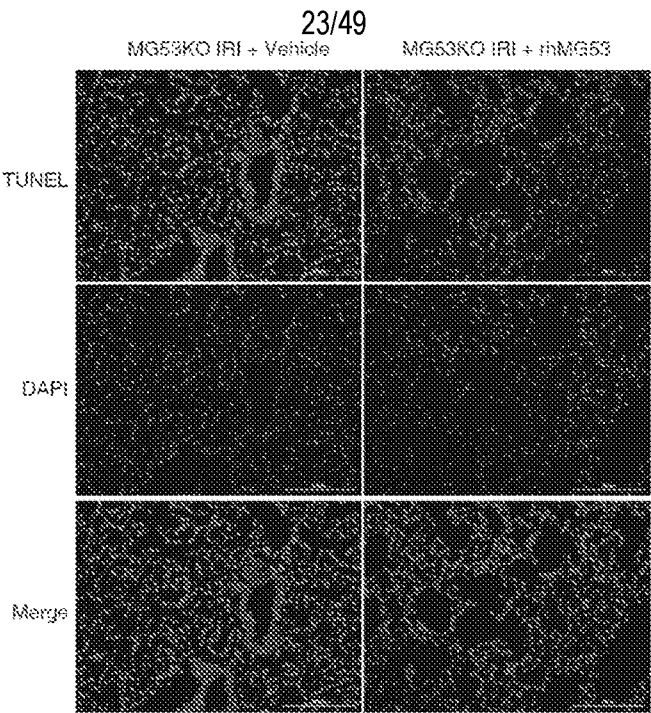


FIG. 22A

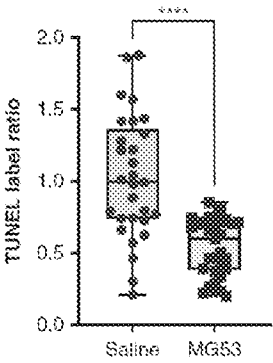


FIG. 22B

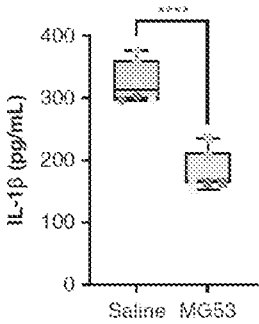


FIG. 23A

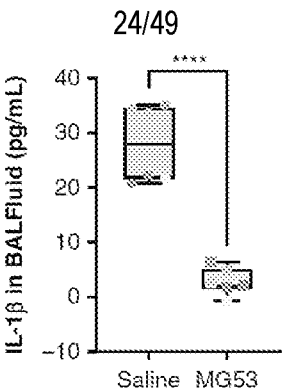


FIG. 23B

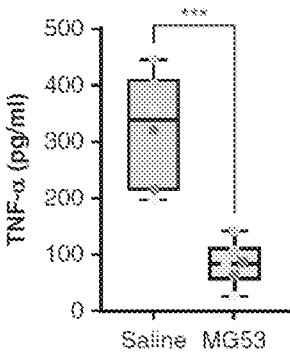


FIG 23C

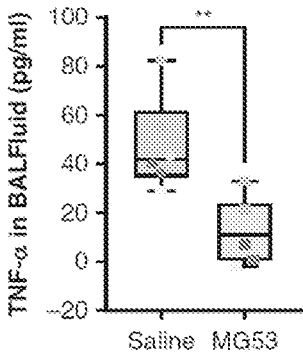


FIG. 23D

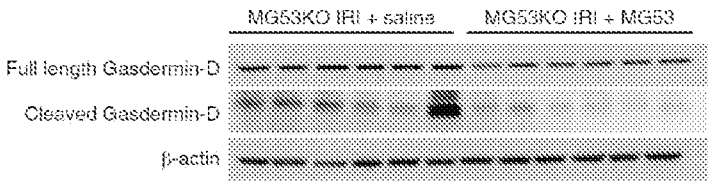


FIG. 24A

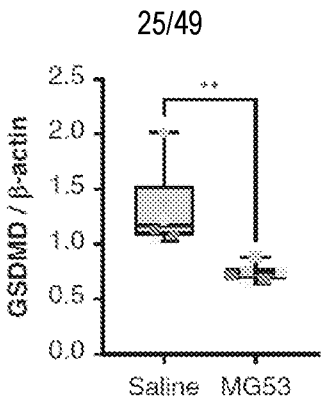


FIG. 24B

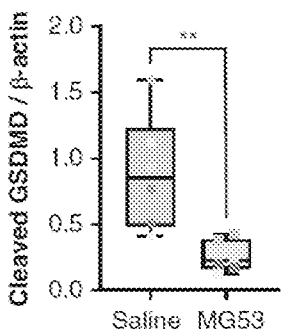


FIG. 24C

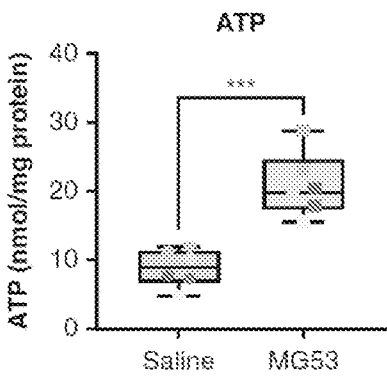


FIG. 24D

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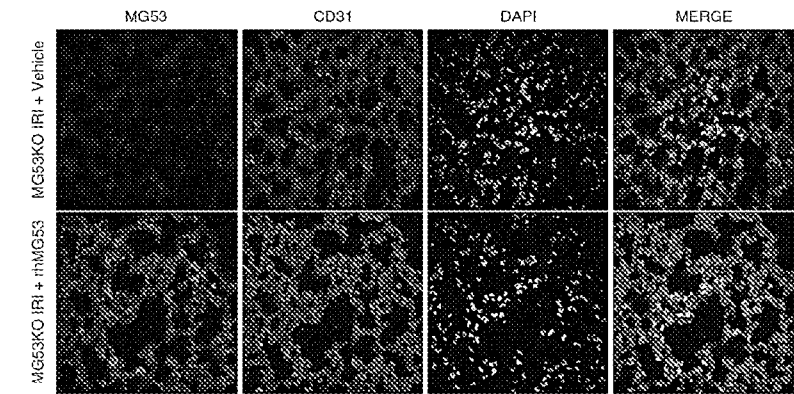


FIG. 25A

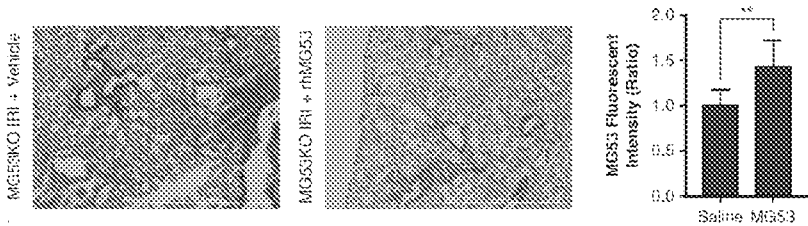


FIG. 25B

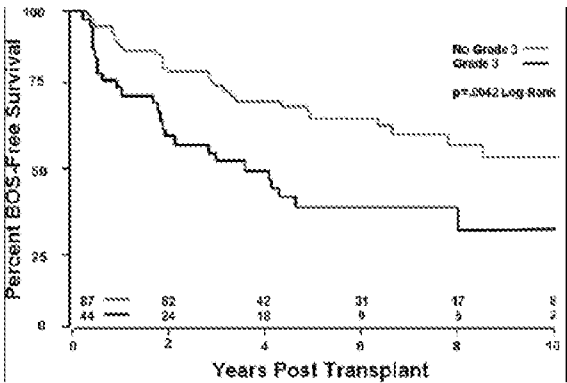


FIG. 26

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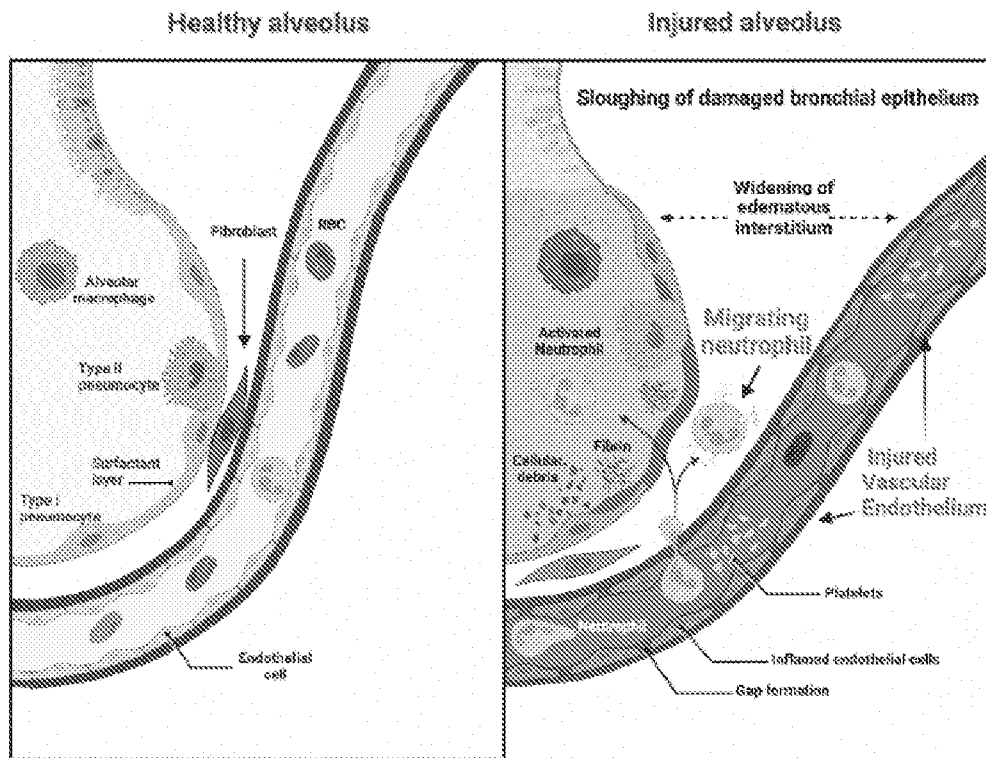


FIG. 27

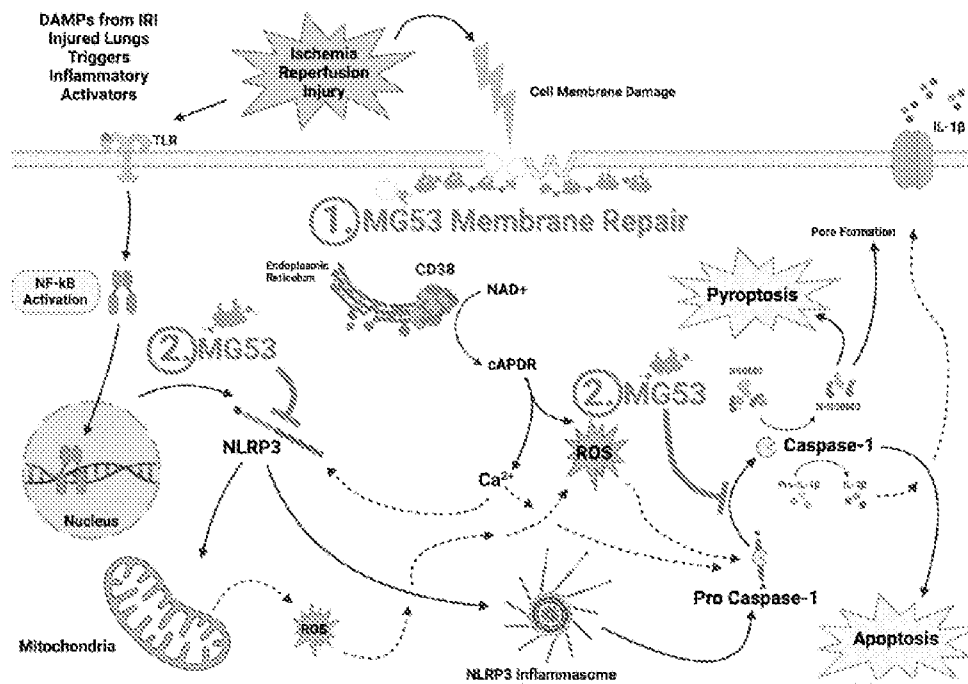


FIG. 28

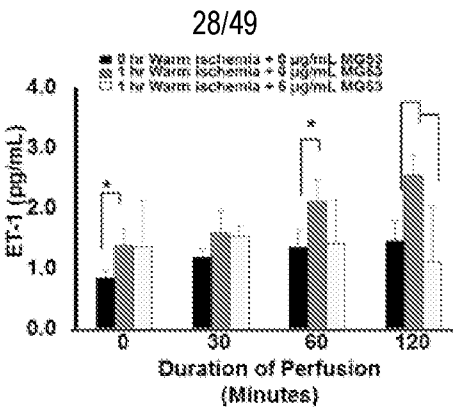


FIG. 29A

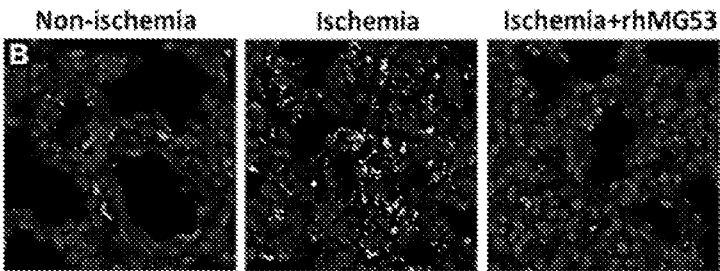


FIG. 29B



FIG. 29C

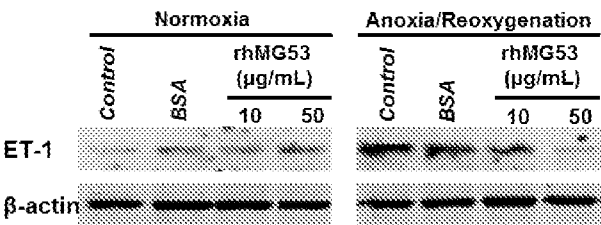


FIG. 29D

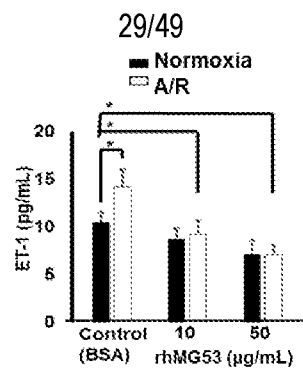


FIG. 29E

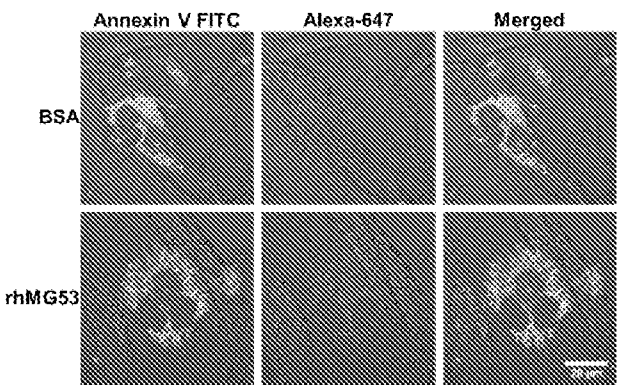


FIG. 30

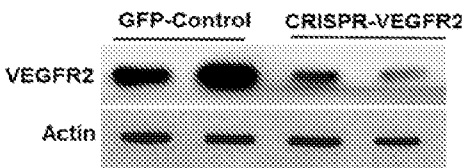


FIG. 31A

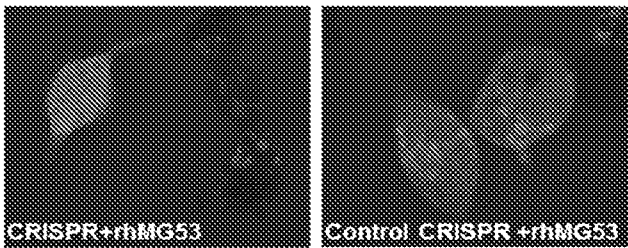


FIG. 31B

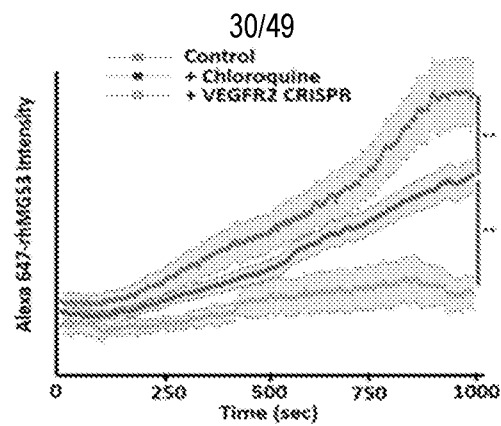


FIG. 31C

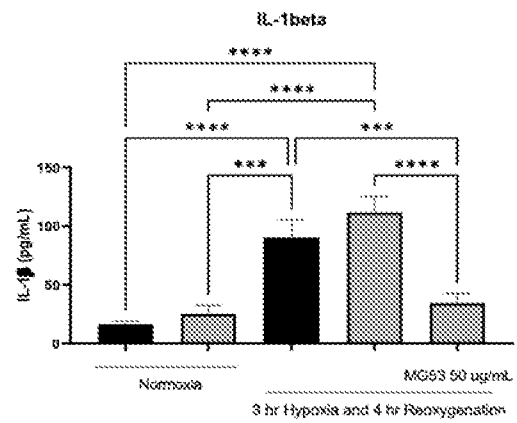


FIG. 32A

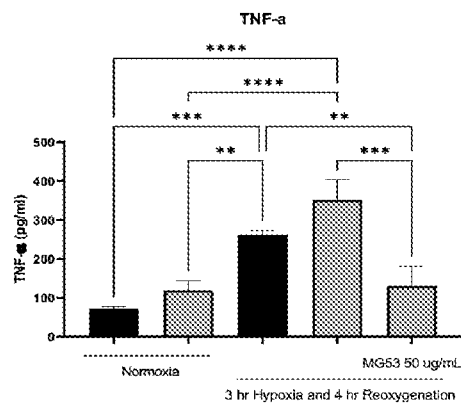


FIG. 32B

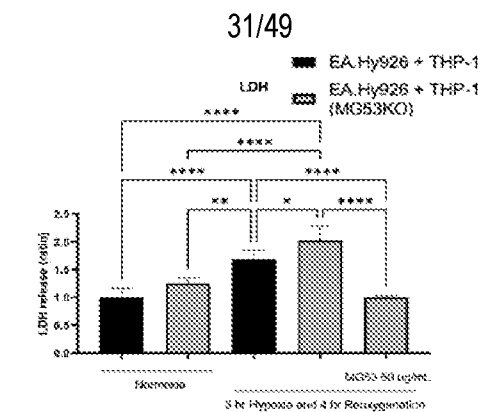


FIG. 32C

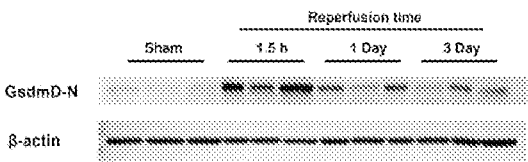


FIG. 33A

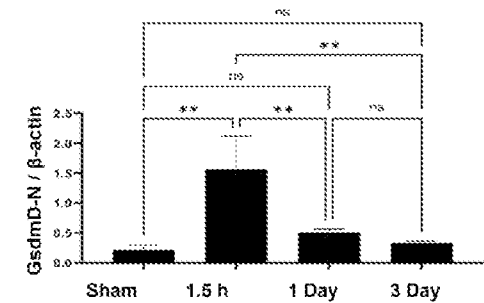


FIG. 33B

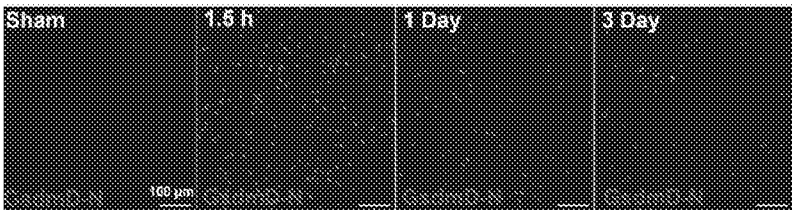


FIG. 33C

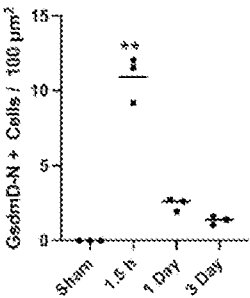


FIG. 33D

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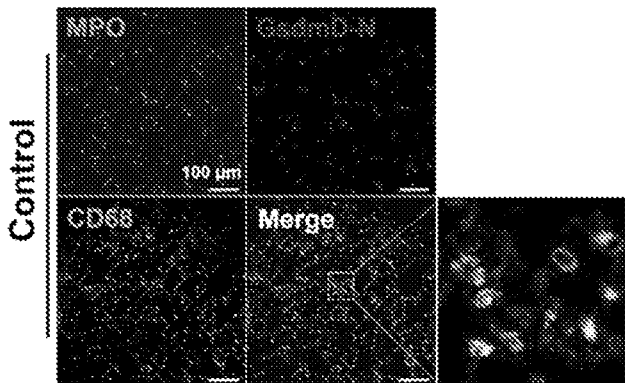


FIG. 34A

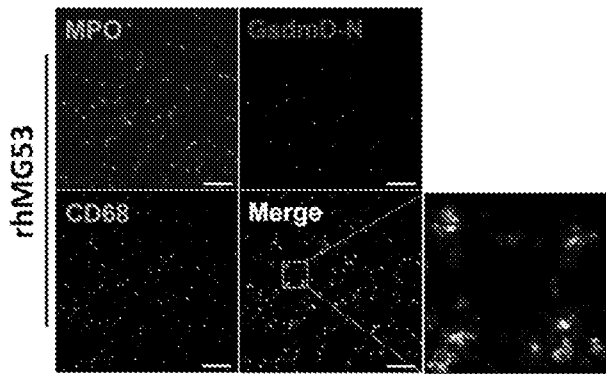


FIG. 34B



FIG. 35

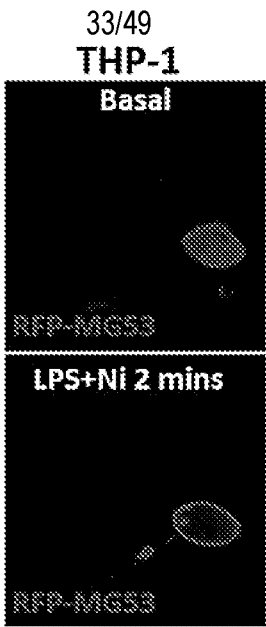


FIG. 36A

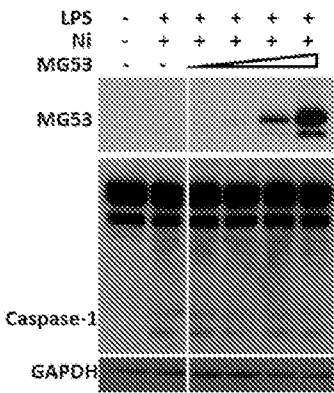


FIG. 36B

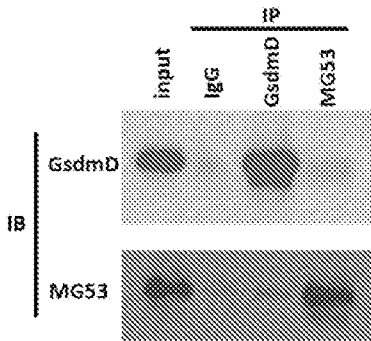


FIG. 37A

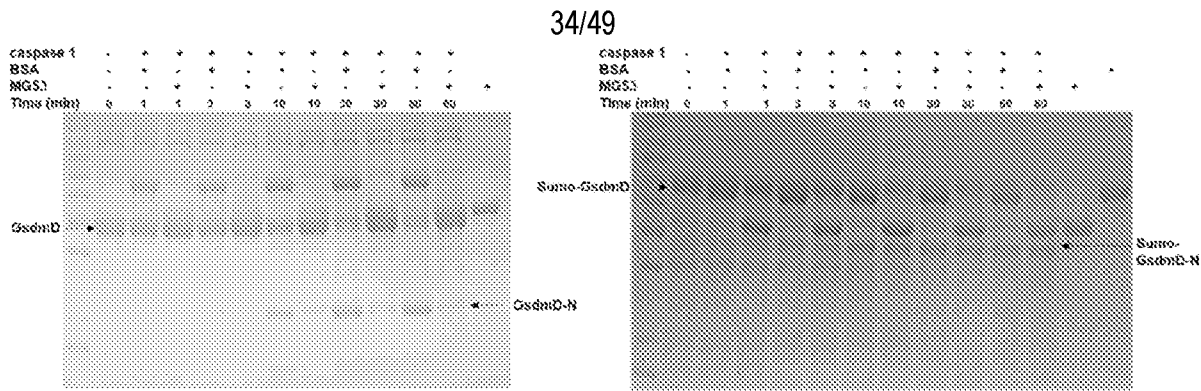


FIG. 37B

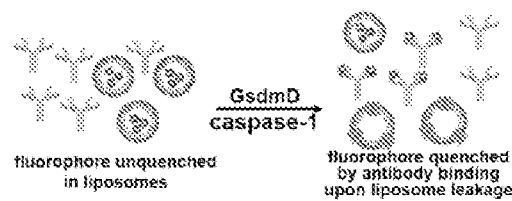


FIG. 38A

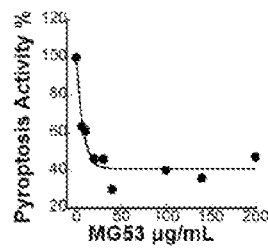


FIG. 38B

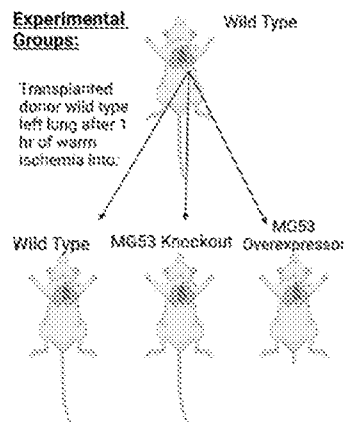


FIG. 39A

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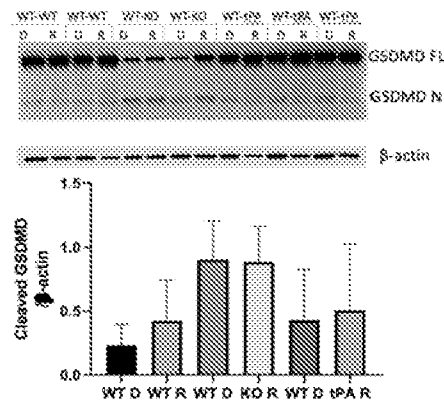


FIG. 39B

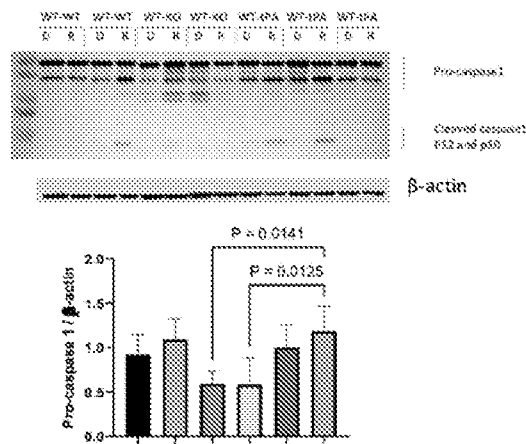


FIG. 39C

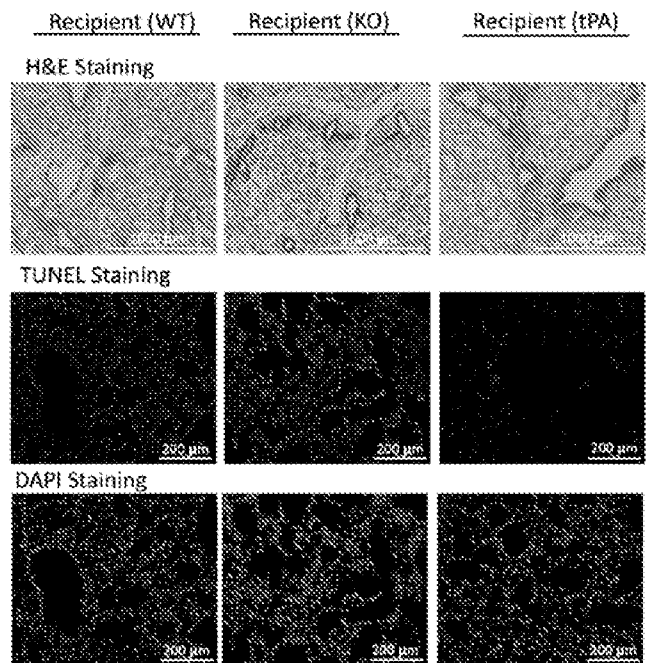


FIG. 39D

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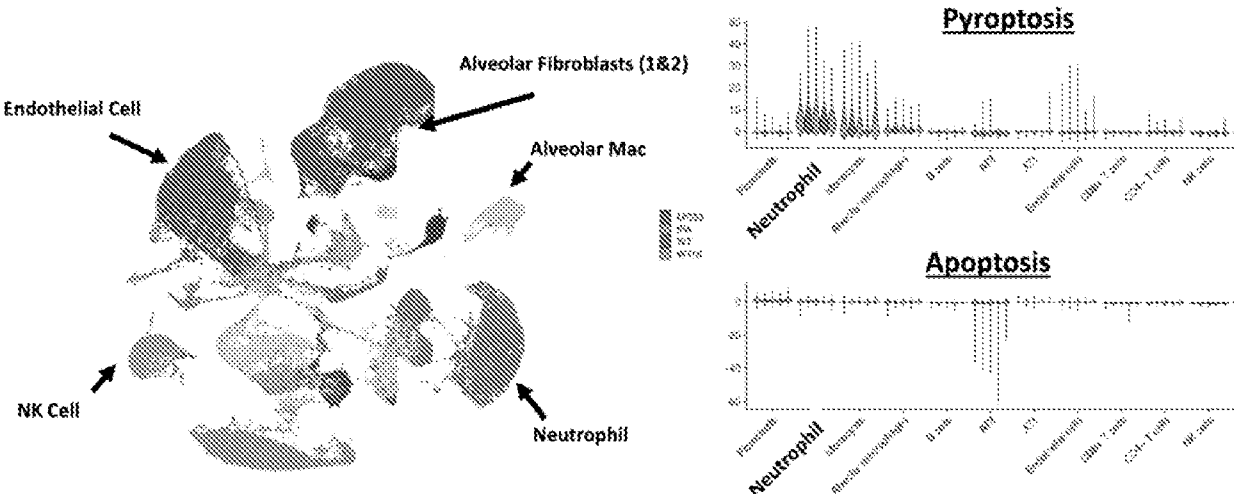


FIG. 40

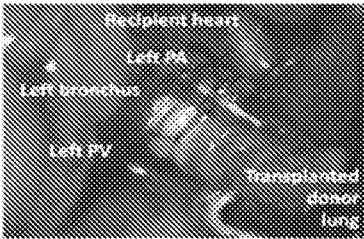


FIG. 41A

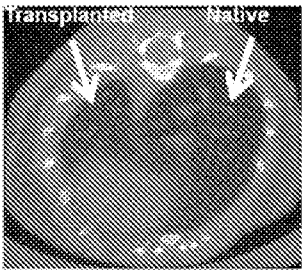


FIG. 41B

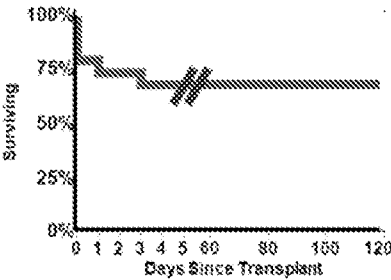


FIG. 41C

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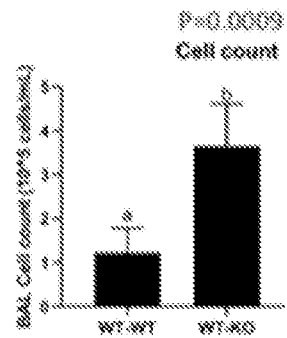


FIG. 41D

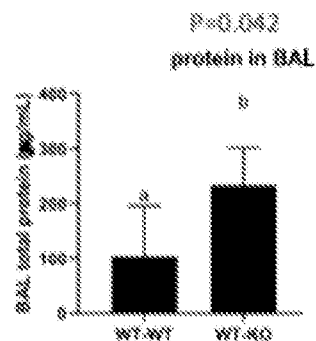


FIG. 41E

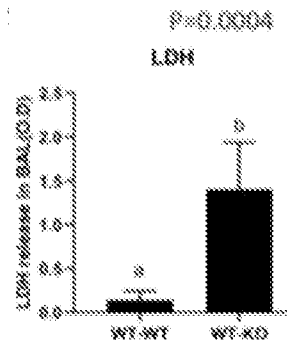


FIG. 41F

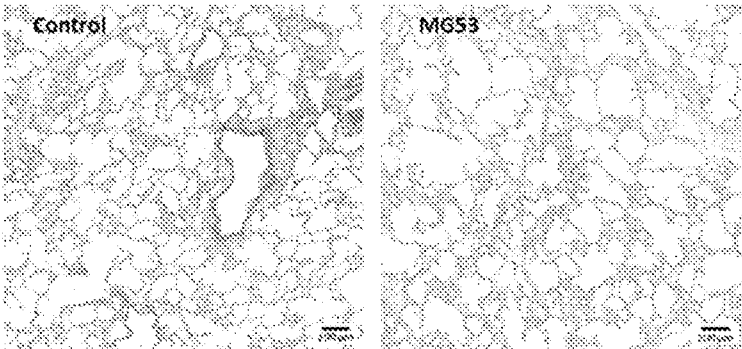


FIG. 42A

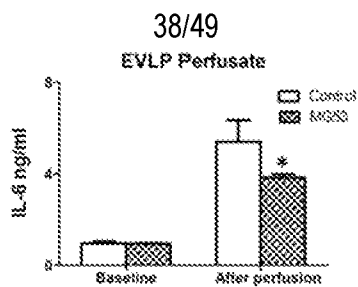


FIG. 42B

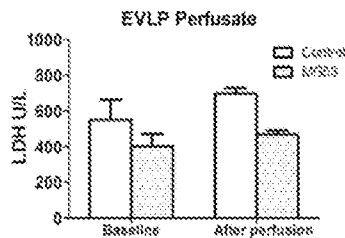


FIG. 42C

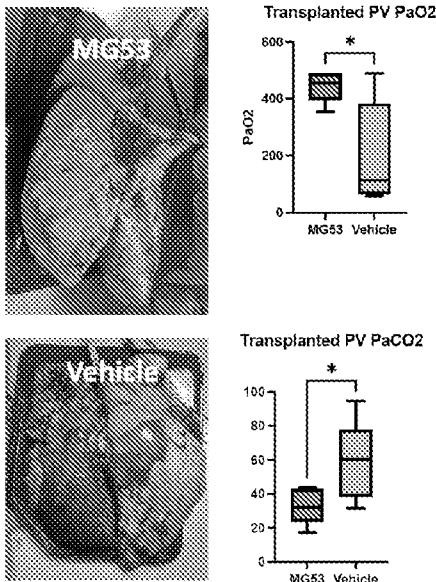


FIG. 43



FIG. 44A

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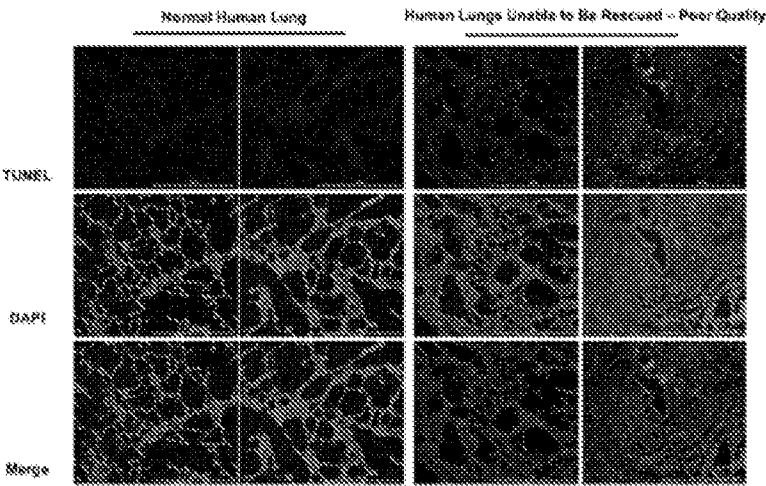


FIG. 44B

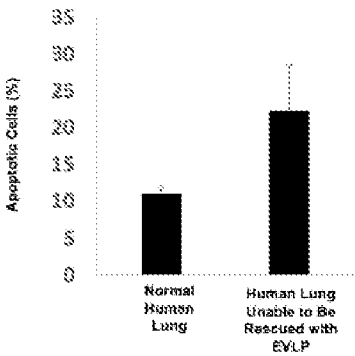


FIG. 44C

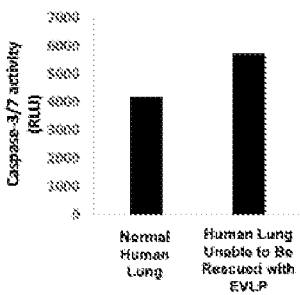


FIG. 44D

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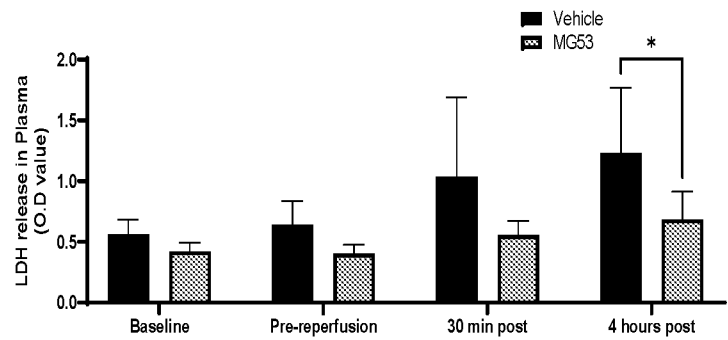


FIG. 45

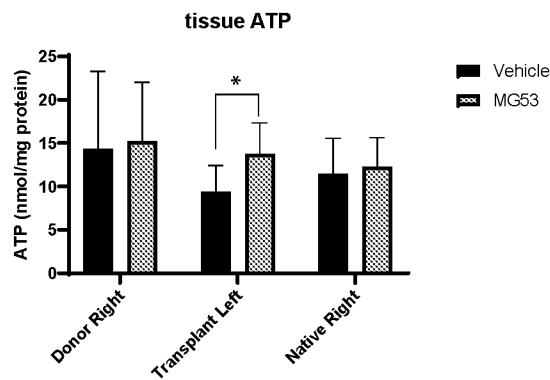


FIG. 46A

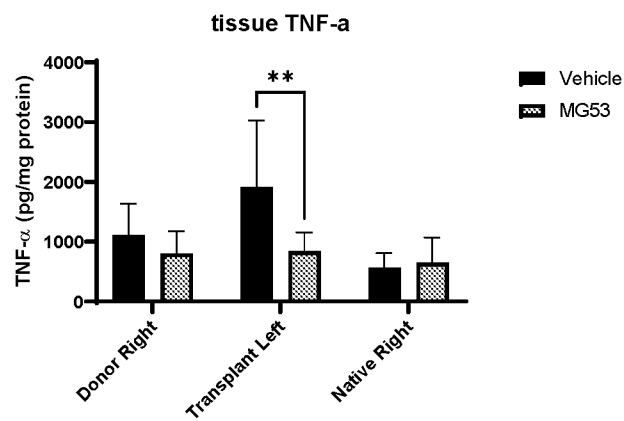


FIG. 46B

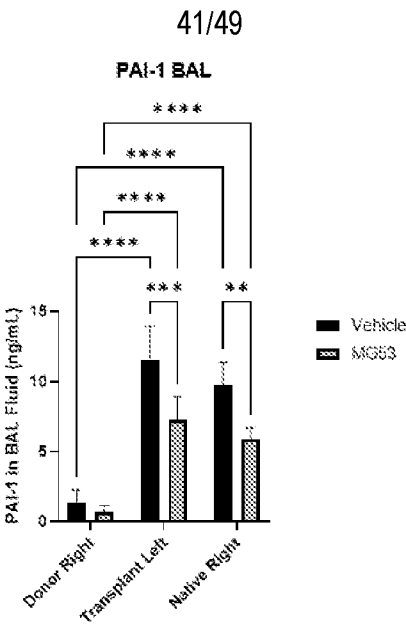


FIG. 47A

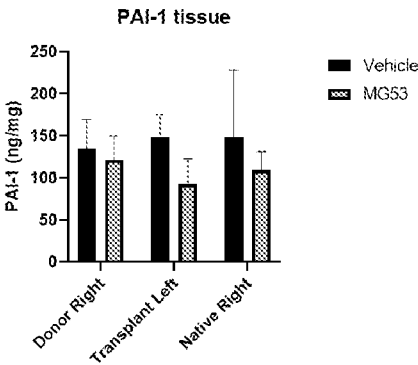


FIG. 47B

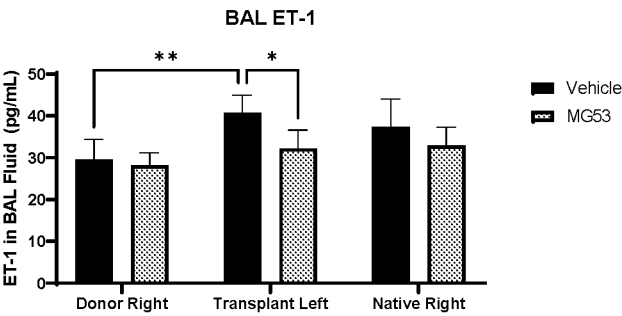


FIG. 48A

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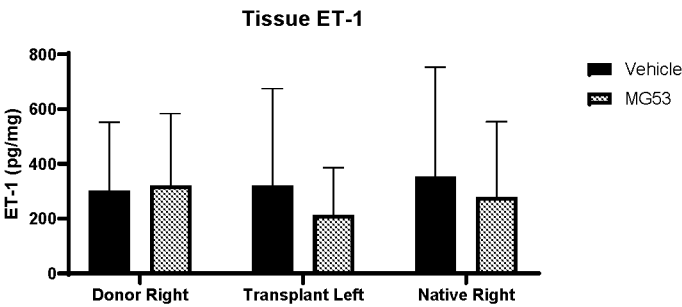


FIG. 48B

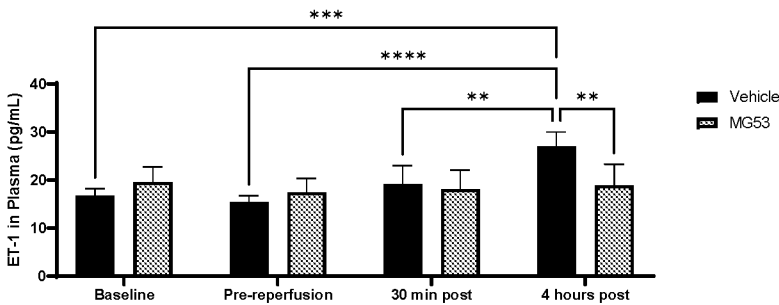


FIG. 48C

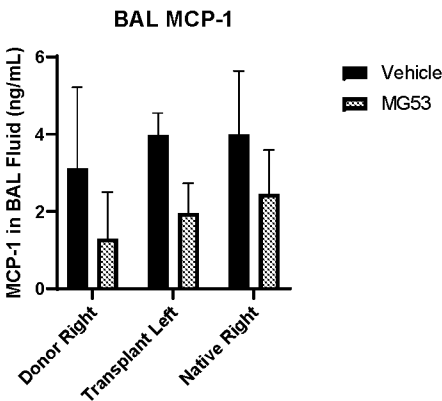


FIG. 49A

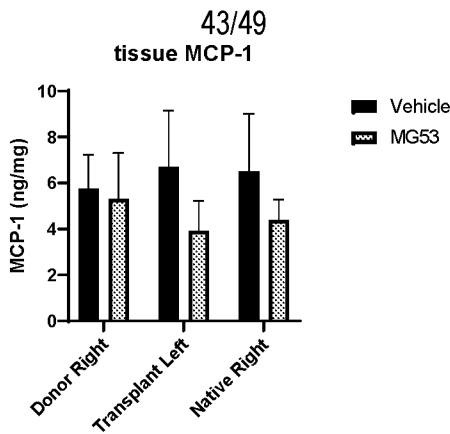


FIG. 49B

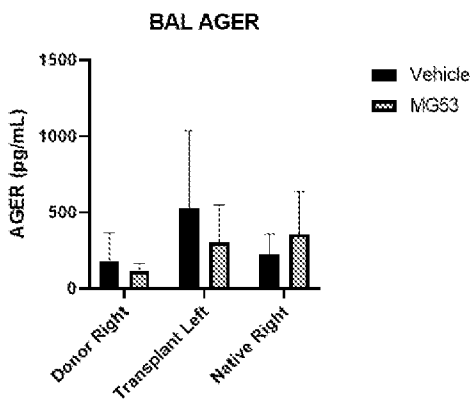


FIG. 50

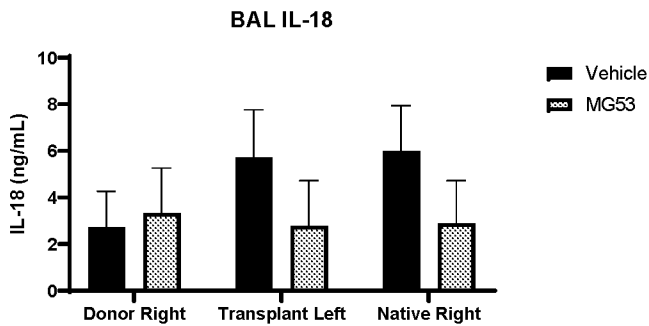


FIG. 51A

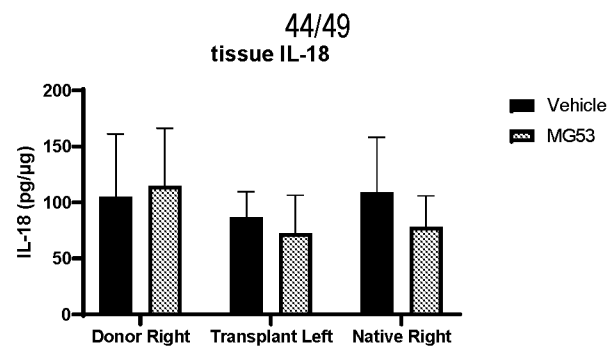


FIG. 51B

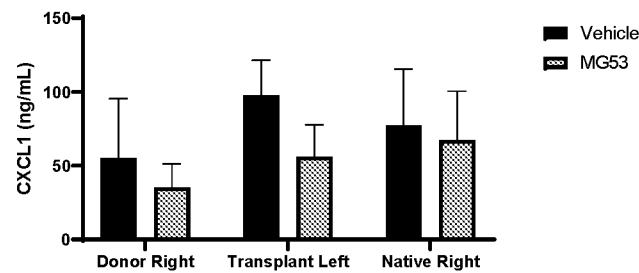


FIG. 52A

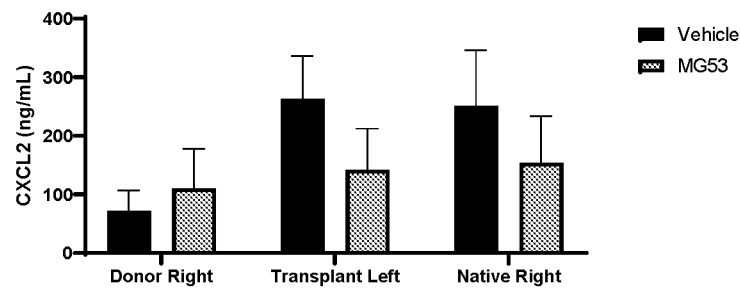


FIG. 52B

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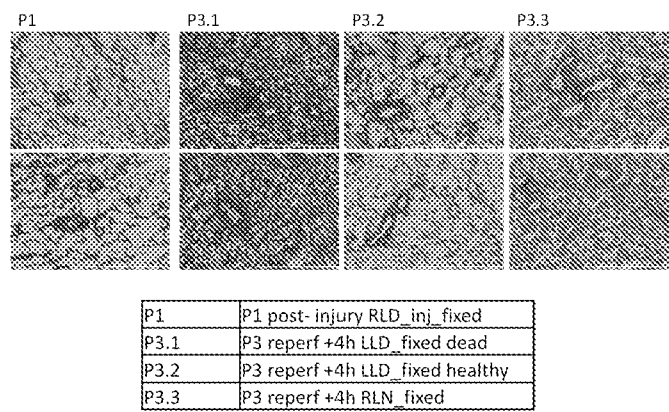


FIG. 53A

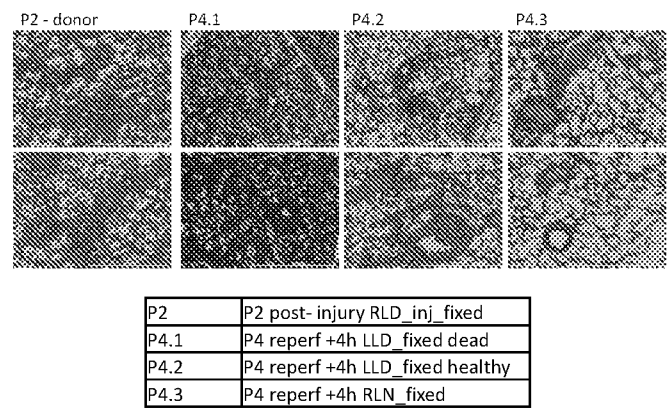


FIG. 53B

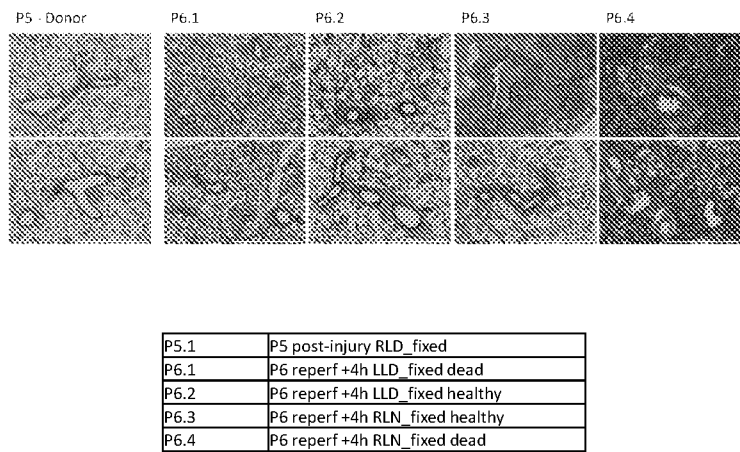
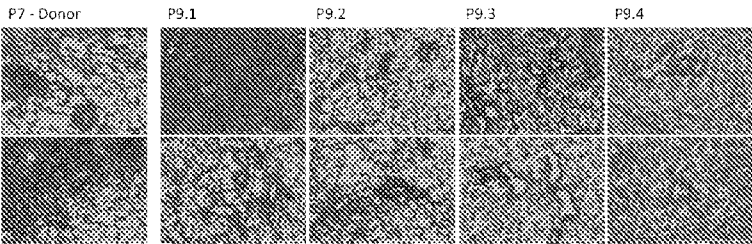


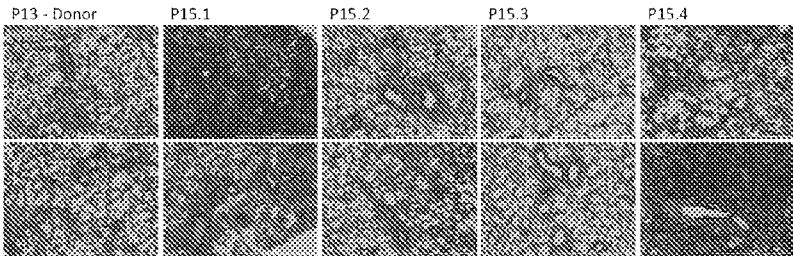
FIG. 54A

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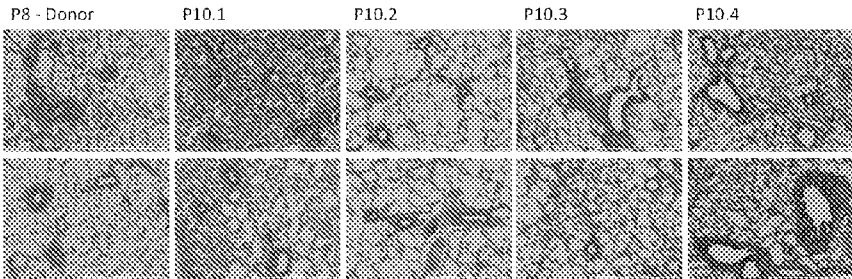
P7.1	P7 post-injury RLD _fixed
P9.1	P9 reperf +4h LLD _fixed dead
P9.2	P9 reperf +4h LLD _fixed healthy
P9.3	P9 reperf +4h RLN _fixed healthy
P9.4	P9 reperf +4h RLN _fixed dead

FIG. 54B



P13.1	P13 post-injury RLD _fixed
P15.1	P15 reperf +4h LLD _fixed dead
P15.2	P15 reperf +4h LLD _fixed healthy
P15.3	P15 reperf +4h RLN _fixed healthy
P15.4	P15 reperf +4h RLN _fixed dead

FIG. 55A



P8.1	P8 post-injury RLD _fixed
P10.1	P10 reperf +4h LLD _fixed dead
P10.2	P10 reperf +4h LLD _fixed healthy
P10.3	P10 reperf +4h RLN _fixed healthy
P10.4	P10 reperf +4h RLN _fixed dead

FIG. 55B

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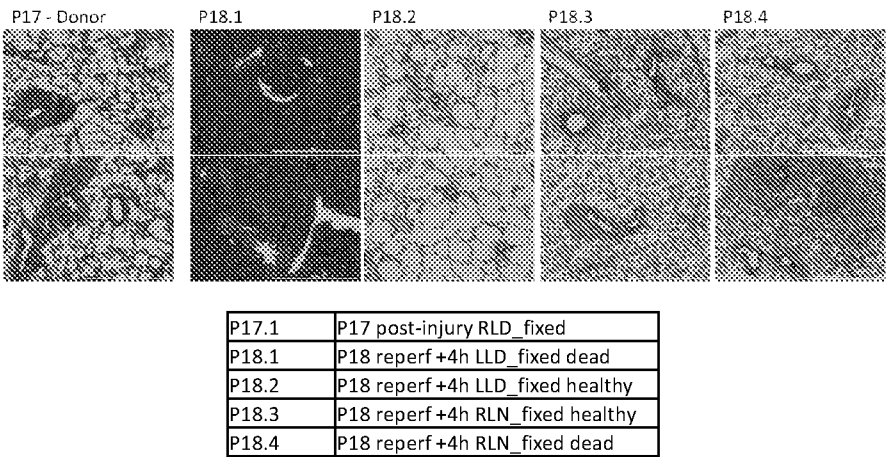


FIG. 56A

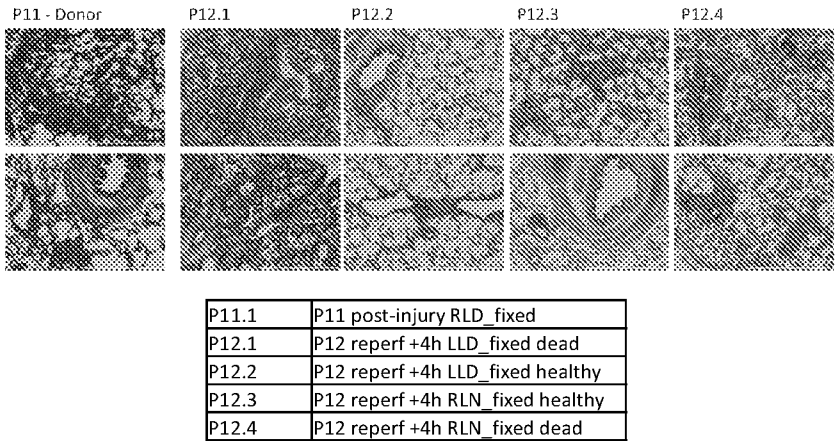


FIG. 56B

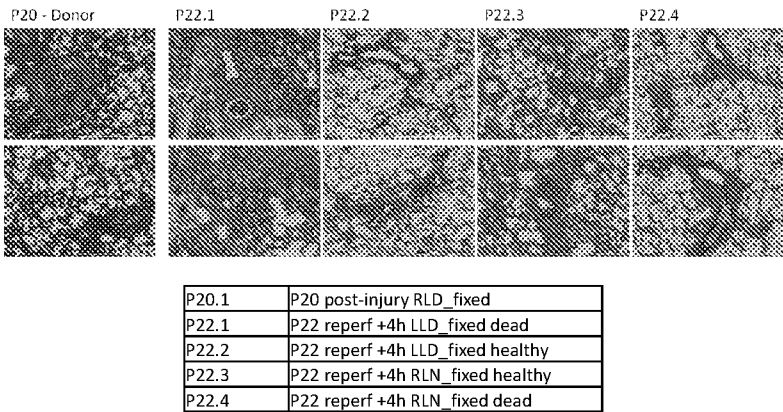


FIG. 57A

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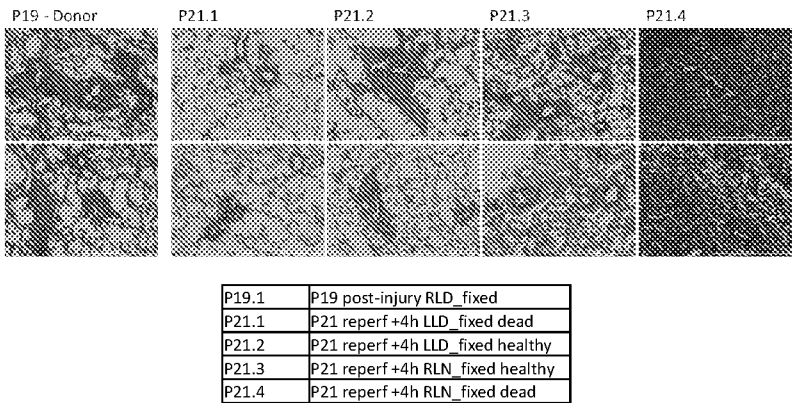


FIG. 57B

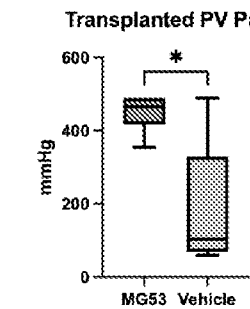


FIG. 58A

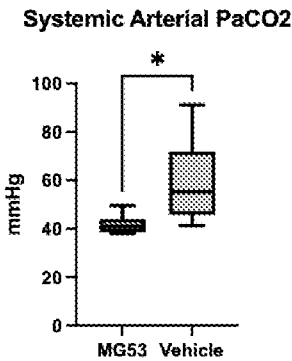


FIG. 58B

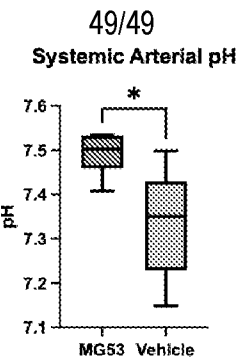


FIG. 58C

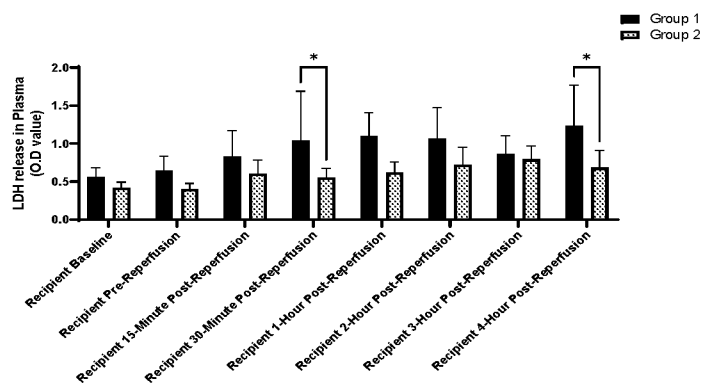


FIG. 58D

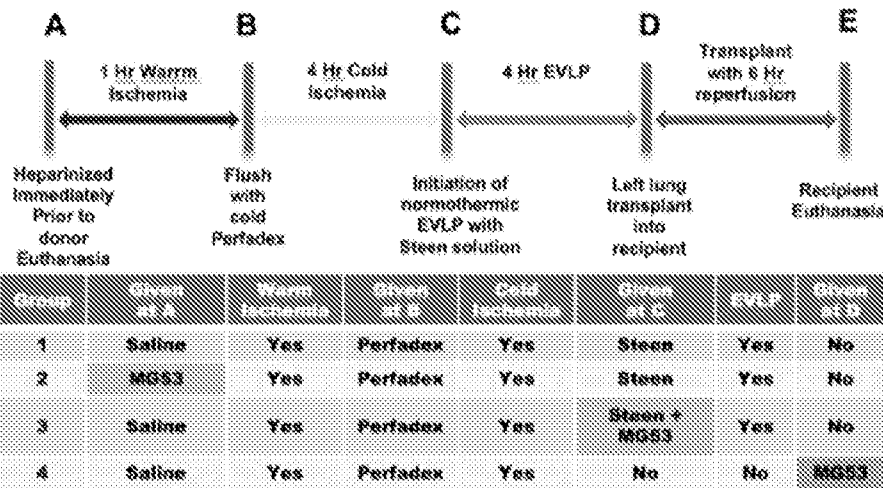


FIG. 59