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(54) Title: PEPTIDE CONJUGATES AND USES THEREOF

(57) Abstract: The present invention relates to relates to peptide conjugates and uses thereof, particularly conjugates comprising a peptide and a superparamagnetic iron oxide nanoparticle (SPION), oral dosage forms comprising the same and uses thereof, for example, in the treatment of fibrosis and fibrosis-related diseases or conditions.

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PEPTIDE CONJUGATES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority from Australian Provisional Patent Application No. 2023902602 filed 16 August 2023, the entire contents of which are incorporated herein by reference.

TECHNICAL FIELD

[0002] The present invention relates to peptide conjugates and uses thereof, particularly conjugates comprising a peptide and a superparamagnetic iron oxide nanoparticle (SPION), oral dosage forms comprising the same and uses thereof, for example, in the treatment of fibrosis and fibrosis-related diseases or conditions.

BACKGROUND

[0003] Peptide-based therapeutics are becoming increasingly popular due to their high specificity and low toxicity. For example, insulin has revolutionised the treatment of diabetes, while various peptide-based therapeutics have been developed for the treatment of cardiovascular, respiratory and neurodegenerative diseases, among others. However, while a number of peptide-based therapeutics have been recognised for being highly selective and efficacious, and relatively safe and well-tolerated in human patients, the clinical application of peptide-based drugs is limited by their short half-lives and plasma stability (as they are commonly broken down by circulating and tissue-resident proteases), and poor oral bioavailability.

[0004] In particular, peptides tend to be poorly orally bioavailable due to their pH sensitivity, leading to breakdown in the gut, as well as their high molecular weight and hydrophilicity, which limits their ability to cross the epithelial barrier in the gastrointestinal tract. As such, most peptide therapeutics are delivered as daily injectable medications or continuously-infused, which is invasive and can be cumbersome. Alternative administration forms are also gaining increasing traction including intranasal and transdermal delivery routes. However, oral delivery remains the preferred route of administration as it can be more convenient, less invasive, more cost-effective and/or involve greater patient

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compliance than other routes of administration, such as intraperitoneal infusion or intranasal administration.

[0005] Accordingly, there is an ongoing need for improved or alternative orally-available peptides for the treatment of a variety of diseases and conditions.

SUMMARY

[0006] In one aspect, the present invention provides a method of orally delivering a peptide to a subject, the method comprising conjugating the peptide to a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) to provide a SPION-peptide conjugate, and orally delivering the SPION-peptide conjugate to the subject.

[0007] In another aspect, the present invention provides use of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a peptide for orally delivering the peptide to a subject.

[0008] In another aspect, the present invention provides a method of treating a disease or condition in a subject, the method comprising orally administering to the subject an effective amount of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a therapeutic peptide, wherein the disease or condition is treatable with the therapeutic peptide.

[0009] In another aspect, the present invention provides a method of diagnosing a disease or condition in a subject, the method comprising orally administering to the subject an effective amount of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a diagnostic peptide, wherein the disease or condition is diagnosable with the diagnostic peptide.

[0010] In another aspect, the present invention provides use of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a therapeutic peptide, in the manufacture of a medicament for treating a disease or condition in a subject by oral administration, wherein the disease or condition is treatable with the therapeutic peptide.

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[0011] In yet another aspect, the present invention provides an oral dosage form comprising a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a peptide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Embodiments of the present invention will now be described with reference to the following Figures, which are intended to be exemplary only, and in which:

[0013] **Figure 1. Schematic overview of how mice were induced to undergo cardiomyopathy over a 2-week period and treated with minipump (Pump)-relaxin (RLX) versus i.p.-administered SPION-RLX versus drinking water (p.o)-administered SPION-RLX from days 7-14 post-injury.** 11-12-week-old male C57BL/6 mice were once-daily administered for 5 consecutive days with either i) saline (vehicle for isoprenaline hydrochloride (ISO); non-injury control group; n=8) or ii) ISO (25mg/kg body weight; injury control group; n=8). Both groups were left untreated for a further 9 days, until day 14. Subgroups of ISO injured mice were subjected to either iii) subcutaneous minipump implantation of 0.5mg/kg/day of recombinant human gene-2 (H2) relaxin on day 7 post-ISO-induced injury (which continuously infused RLX into the circulation of treated mice for a 7 day period; from days 7-14; positive treatment control; ISO+ Pump-RLX group; n=8); iv) 25ng/day of SPION-RLX via i.p injections every 72 hours (on days 7, 10 and 13; ISO+SPION-RLX (i.p) group; n=8); v) 25ng/day SPION-RLX through daily drinking water from days 7-14 post-ISO injury (ISO+SPION-RLX (p.o) group; n=8); or vi) empty SPIONs that were not conjugated to RLX either i.p every 72 hours (n=4) or through daily drinking water administration (n=4) (ISO+ Empty-SPION vehicle control group). All mice underwent cardiac ultrasound measurements (for assessment of cardiac function) before being killed for tissue collection and analysis on day 14.

[0014] **Figure 2. Schematic overview of how mice were induced to undergo cardiomyopathy over a 6-week period and treated with orally (p.o)-administered SPION-RLX or SPION-B7-33 or unconjugated RLX or B7-33 or the angiotensin converting enzyme (ACE) inhibitor, Perindopril, from days 14-42 post-injury.** 11-12-week-old male C57BL/6 mice were once-daily administered for 5 consecutive days with either i) saline (vehicle for isoprenaline hydrochloride (ISO); non-injury control group; n=7)

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or ii) ISO (25mg/kg body weight; injury control group; n=7). Both groups were left untreated for a further 37 days, until day 42. Subgroups of ISO injured mice were subjected to oral gavage (p.o) administration, every 3 days from day 14 to day 42 (4-week period) of either iii) SPION-RLX (25ng/day; n=7); iv) SPION-B7-33 (25ng/day; n=7); v) Perindopril (60ng/day; n=7); vi) unconjugated RLX (25ng/day; n=7); vii) unconjugated B7-33 (25ng/day; n=7) or viii) Empty SPIONs alone (n=7). All mice were killed for tissue collection and analysis on day 42.

[0015] **Figure 3. Schematic overview of how mice were induced to undergo acute lung injury over a 7-day period and treated with intranasal (i.n)-administered SPION-RLX from days 2-6 post-injury.** 6-8-week-old female BALB/c mice were given an intranasal (i.n) instillation of either i) saline (50ml; vehicle for lipopolysaccharide (LPS); non-injury control group; n=6) or ii) LPS (50ml of a 5mg/ml solution; 250ng/mouse; injury control group; n=6). Both groups were left untreated until day 7. Subgroups of LPS injured mice were subjected to an i.n instillation of either iii) SPION-RLX (25ng in 50ml/day) or iv) Empty-SPIONs (50ml/day) on days 2, 4 and 6 post-LPS injury. All mice underwent plethysmography (for assessment of airway/lung function) before being killed for tissue collection and analysis on day 7.

[0016] **Figure 4. The effects of minipump (Pump)-infused relaxin (RLX) versus i.p.-administered SPION-RLX versus drinking water (p.o)-administered SPION-RLX on measures of myocardial/left ventricular (LV) inflammation in mice with ISO-induced cardiomyopathy, when delivered from days 7-14 post-ISO injury.** Repeated ISO (25mg/kg over 5 consecutive days) administration to mice induced a significant increase in inflammatory cell infiltration (a,b), and inflammatory cytokine expression including tumour necrosis factor (TNF)-a (c) and interleukin (IL)-1b (d), but reduced CD11c⁺ dendritic cell (DC) (e,f) and CD11c⁺ CD206⁺ DC (e,g) infiltration into the LV at 14-days post-injury, compared to respective measurements obtained from saline (SAL)-treated control mice. (a) Representative images of hematoxylin and eosin (H&E)-stained LV tissue sections, show the extent of LV inflammatory cell infiltration (as visualised by the number of purple-stained cell nuclei) in each of the groups investigated. (b-d) Also shown are the mean $\pm$ standard error of the mean (SEM) number of inflammatory cells per field (as determined by morphometric analysis of 10 random and non-overlapping fields of view per section) (b),

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LV TNF- α levels (c) and LV IL-1 β levels (d) (as determined by ELISA assays of LV protein extracts (c,d)). (e) Representative flow cytometry-derived fluorescence-activated cell sorting (FACS) plots show the extent of CD11c⁺ DC infiltration within LV tissue from each of the groups indicated. (f,g) Also shown are the mean $\pm$ standard error of the mean (SEM) LV CD11c⁺ DCs (f) and CD11c⁺ CD206⁺ DCs (g) in each of the groups investigated. The intraperitoneal (i.p) administration of SPION-RLX (25ng/day; on days 7, 10 and 13 post-ISO-injury) or daily drinking water (p.o) administration of SPION-RLX (25ng/day; from days 7-14 post-ISO-injury) was able to reduce the ISO-induced increase in LV inflammatory cell infiltration (b), LV TNF- α levels (c) and LV IL-1 β levels (d), to the same extent as Pump-infused RLX (0.5mg/kg/day; from days 7-14 post-ISO-injury), at day 14-post-injury. Furthermore, the i.p or p.o administration of SPION-RLX was able to restore, and even increase, the extent of LV CD11c⁺ DC (f) and CD11c⁺ CD206⁺ DC (g) infiltration into the LV, to an equivalent extent as Pump-infused RLX, at day-14 post-injury (after 7 days of treatment). However, the Empty-SPIONs alone were not able to achieve any of the effects of i.p-administered or p.o-administered SPION-RLX. The data presented in panels b-d were obtained from n=7-8 mice per group; whereas the data from panels f,g were obtained from n=3 separate assays (tissue pooled from n=2-3 mice per assay; from the n=7-8 mice) per group. The white coloured circles in each of the bar plots represent the individual data points per group. *P<0.05, **P<0.01, ***P<0.001 versus the saline group; #P<0.05, ##P<0.01, ###P<0.001 versus the ISO group; ¶P<0.05, ¶¶P<0.01, ¶¶¶P<0.001 versus the ISO+Empty-SPION-treated group; as determined by a one-way ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0017] **Figure 5. The effects of minipump (Pump)-infused relaxin (RLX) versus i.p-administered SPION-RLX versus drinking water (p.o)-administered SPION-RLX on myocardial/left ventricular (LV) M2-like macrophage and regulatory T cell (T_{reg}) infiltration in mice with ISO-induced cardiomyopathy, when delivered from days 7-14 post-ISO injury.** Repeated ISO (25mg/kg over 5 consecutive days) administration to mice induced a significant increase in M2-like macrophage and T_{reg} infiltration into the LV, the latter being a known promoter of fibrosis. (a,b) Representative flow cytometry-derived fluorescence-activated cell sorting (FACS) plots show the extent of F4/80⁺ CD206⁺ positive M2-like macrophages (a) and CD4⁺ CD25⁺ FoxP3-positive T_{regs} (b) in each of the groups indicated. Also shown is the mean $\pm$ standard error of the mean (SEM) proportion of F4/80⁺

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CD206⁺ positive M2-like macrophages (a) or CD4⁺ CD25⁺ FoxP3-positive T_{regs} (b) in each of the groups investigated. Only Pump-RLX treatment significantly reduced the ISO-induced increase in M2-like macrophage infiltration (a). However, the ISO-induced increase in LV T_{reg} infiltration was equivalently reduced by either Pump-infused RLX (0.5mg/kg/day; from days 7-14 post-ISO-injury), intraperitoneal (i.p)-administered SPION-RLX (25ng/day; on days 7, 10 and 13 post-ISO-injury) or daily drinking water (p.o)-administered SPION-RLX (25ng/day; from days 7-14 post-ISO-injury) but not Empty-SPIONs (administered either through i.p injections or daily drinking water from days 7-14 post-injury), at day-14 post-injury. In each case (a,b), the data presented were obtained from n=3 separate assays (tissue pooled from n=2-3 mice per assay; from n=7-8 mice in total) per group. The white coloured circles in each of the bar plots represent the individual data points per group. *P<0.05, **P<0.01 versus the saline group; #P<0.05 versus the ISO group; as determined by a one-way ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0018] **Figure 6. Plasma relaxin levels from mice with ISO-induced cardiomyopathy and treated with minipump (Pump)-infused relaxin (RLX) versus i.p-administered SPION-RLX versus drinking water (p.o)-administered SPION-RLX from days 7-14 post-injury.** Shown are the mean $\pm$ standard error of the mean (SEM) plasma (H2) relaxin (RLX) levels from mice subjected to repeated ISO (25mg/kg over 5 consecutive days) administration, and treated with either Pump-infused RLX (0.5mg/kg/day; from days 7-14 post-ISO-injury) or intraperitoneal (i.p)-administered SPION-RLX (NP-RLX; 25ng/day; on days 7, 10 and 13 post-ISO-injury) or daily drinking water (p.o)-administered SPION-RLX (NP-RLX; 25ng/day; from days 7-14 post-ISO-injury) or Empty-SPIONs (administered either through i.p injections or daily drinking water from days 7-14 post-injury), at day-14 post-injury. Data were obtained from n=7-8 mice per group; where the white coloured circles in each of the bar plots represent the individual data points per group.

[0019] **Figure 7. The effects of intranasal (i.n)-instilled SPION-relaxin (RLX) on measures of airway/lung inflammation in mice with LPS-induced acute lung injury, when delivered from days 2-6 post-LPS injury.** LPS (250ng/mouse; i.n) administration to mice induced a significant increase in inflammatory cell infiltration within the airways (a,b) and airway emphysema (c); lung inflammatory cytokine expression including tumour

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necrosis factor (TNF)-a (**d**), interleukin (IL)-1b (**e**) and IL-6 (**f**); airway epithelial damage (**g**); CD11c⁺ dendritic cell (DC) (**h,i**) and F4/80⁺ CD206⁻ M1-like macrophage infiltration (**k,l**); but reduced F4/80⁺ CD206⁺ M2-like macrophage infiltration (**k,m**) within the airways/lung at 7-days post-injury. (**a**) Representative images of hematoxylin and eosin (H&E)-stained lung tissue sections, show the extent of airway/lung inflammatory cell infiltration and widening of the alveolar space (which is a feature of emphysema) in each of the groups investigated. (**b,c**) Also shown are the mean $\pm$ standard error of the mean (SEM) number of inflammatory cells per field (**b**) and mean linear intercept (MLI) index (as an indication of emphysema score; **c**) (as determined by the morphometric analysis of 10 random and non-overlapping fields of view per section in each case). (**d-g**) Also shown is the mean $\pm$ SEM lung TNF-a (**d**), IL-1b (**e**) and IL-6 (**f**) expression levels as well as thymic stromal lymphopoietin (TSLP)-stained epithelial damage (**g**) in the airways/lungs of each of the groups analysed (as determined by the morphometric analysis of 10 random and non-overlapping fields of view per section in each case). (**h,k**) Representative flow cytometry-derived fluorescence-activated cell sorting (FACS) plots show the extent of CD11c⁺ DC (**h**) or F4/80⁺ CD206 macrophage (**k**) infiltration within the airways/lung of the groups indicated. (**i,j,l,m**) Additionally shown are the mean $\pm$ SEM airway/lung CD11c⁺ DC (**i**), F4/80⁺ CD206⁻ M1-like macrophage (**l**) or F4/80⁺ CD206⁺ M2-like macrophage (**m**) infiltration; and CD80 mean fluorescence intensity of DC uptake (**j**) in each of the groups investigated. The i.n-administration of SPION-RLX (25ng/day; on days 2, 4 and 6 post-LPS-injury) significantly attenuated the LPS-induced increase in airway/lung inflammatory cell infiltration (**b**), emphysema (**c**), TNF-a levels (**d**), IL-1b levels (**e**) IL-6 levels (**f**), airway epithelial damage (**g**), DC (**i**) and M1-like macrophage (**l**) infiltration; but increased M2-like macrophage infiltration (**m**) at day 7-post injury. SPION-RLX was also found to be taken up by infiltrating DCs within the airway/lung (**j**). However, the Empty-SPIONs alone were not able to achieve any of the effects of i.n-administered SPION-RLX, and were not taken up by infiltrating DCs. The data presented in panels **b-g** were obtained from n=5-6 mice per group; whereas the data from panels **i,j,l,m** were obtained from n=3 separate assays (tissue pooled from n=2 mice per assay; from the n=6 mice) per group. The white coloured circles in each of the bar plots represent the individual data points per group. *P<0.05, **P<0.01, ***P<0.001 versus the saline group; #P<0.05, ###P<0.001 versus the LPS group; ¶P<0.05, ¶¶P<0.001 versus the ISO+Empty-SPION-treated group; as determined by a one-way

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ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0020] **Figure 8. Infiltrating dendritic cells (DCs) and M1-like macrophages within the airways/lung were found to express the relaxin (RLX) receptor, Relaxin Family Peptide Receptor 1 (RXFP1) and were involved in the uptake of SPION-RLX in mice with LPS-induced acute lung injury.** (a-c) Representative flow cytometry-derived fluorescence-activated cell sorting (FACS) plots show that infiltrating CD11c⁺ dendritic cells (DCs) (a,b) and F4/80⁺ CD206⁻ M1-like macrophages (a,c) within the airways/lung expressed RXFP1 and were involved in the uptake of i.n-administered fluorescein isothiocyanate (FITC)-labelled RLX conjugated to SPIONs (SPION-RLX). (d) Also shown is the immunohistochemical localisation of SPION-RLX (brown staining) in DCs and M1-like macrophages within the airways/lung of LPS-injured mice i.n.-treated with SPION-RLX. (e) Additionally shown is the mean $\pm$ SEM airway reactivity (airway hyperresponsiveness; as measured by invasive plethysmography) in each of the groups investigated, in response to increasing concentrations of the bronchoconstrictor, methacholine (3.125-50mg/ml). The i.n-administration of SPION-RLX (25ng/day; on days 2, 4 and 6 post-LPS-injury), but not Empty-SPIONs, significantly attenuated the LPS-induced airway reactivity/hyperresponsiveness back to levels measured in saline (SAL)-treated control mice at day 7-post injury. The data presented in panel e were obtained from n=5-6 mice per group. **P<0.01, ***P<0.001 versus the saline group; ###P<0.001 versus the LPS group; ¶P<0.01 versus the ISO+Empty-SPION-treated group; as determined by a two-way ANOVA and Bonferoni's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0021] **Figure 9. The effects of minipump (Pump)-infused relaxin (RLX) versus i.p-administered SPION-RLX versus drinking water (p.o)-administered SPION-RLX on measures of myocardial/left ventricular (LV) fibrosis, hypertrophy and vascular rarefaction in mice with ISO-induced cardiomyopathy, when delivered from days 7-14 post-ISO injury.** Repeated ISO (25mg/kg over 5 consecutive days) administration to mice induced a significant increase in interstitial LV fibrosis (a,b), myofibroblast accumulation (c), pro-fibrotic transforming growth factor (TGF)-b1 (d), cardiomyocyte hypertrophy (enlargement; e,f) and vascular rarefaction (loss of blood vessel density; g,h),

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within the LV at 14-days post-injury, compared to respective measurements obtained from saline (SAL)-treated control mice. (a) Representative images (with enlarged insets) of picrosirius red-stained LV tissue sections, show the extent of interstitial collagen (red) deposition within the LV midzone in each of the groups investigated. (b-d) Also shown are the mean $\pm$ standard error of the mean (SEM) % interstitial LV collagen deposition (fibrosis) per field (as determined by morphometric analysis of 10 random and non-overlapping fields of view per section; b), number of interstitial myofibroblasts per field (c) and interstitial LV TGF- β 1 levels per field (d) (as determined by morphometric analysis of 10 random and non-overlapping fields of view per immunohistochemically stained section in each case). (e) Representative picrosirius red-stained images also show the extent of LV cardiomyocyte size in each of the groups investigated. (f) The mean $\pm$ SEM LV cardiomyocyte cross sectional area in each of the groups analysed was then determined (from 100 cardiomyocytes in 10 random and non-overlapping fields of view per section). (g) Representative immunohistochemically-stained sections for α -smooth muscle actin (SMA), show the extent of α -SMA-stained blood vessel density in each of the groups investigated. (h) Also shown is the mean $\pm$ SEM LV blood vessel density per group analysed (from the morphometric analysis of 10 random and non-overlapping fields of view per). The intraperitoneal (i.p) administration of SPION-RLX (NP-RLX; 25ng/day; on days 7, 10 and 13 post-ISO-injury) or daily drinking water (p.o) administration of SPION-RLX (NP-RLX 25ng/day; from days 7-14 post-ISO-injury) was able to reduce the ISO-induced increase in interstitial LV collagen deposition (fibrosis) (b), interstitial LV myofibroblast accumulation (c), interstitial LV IL-1b levels (d) and LV cardiomyocyte size (e), to the same extent as Pump-infused RLX (0.5mg/kg/day; from days 7-14 post-ISO-injury), at day 14-post-injury. Furthermore, the i.p or p.o administration of SPION-RLX (NP-RLX) was able to restore, the ISO-induced loss of blood vessel density to an equivalent extent as Pump-infused RLX, at day-14 post-injury (after 7 days of treatment). However, the Empty-SPIONs (Empty-NPs) alone were not able to achieve any of the effects of i.p-administered or p.o-administered SPION-RLX (NP-RLX). The data presented in panels b-d, f and h were obtained from n=6-8 mice per group. The white coloured circles in each of the bar plots represent the individual data points per group. *P<0.05, **P<0.01, ***P<0.001 versus the saline group; #P<0.05, ##P<0.01, ###P<0.001 versus the ISO group; [†]P<0.05, ^{††}P<0.01, ^{†††}P<0.001 versus the ISO+Empty-

SPION-treated group; as determined by a one-way ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0022] **Figure 10. The effects of minipump (Pump)-infused relaxin (RLX) versus i.p-administered SPION-RLX versus drinking water (p.o)-administered SPION-RLX on measures of extracellular matrix (ECM) degradation: matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in mice with ISO-induced cardiomyopathy, when delivered from days 7-14 post-ISO injury.** Repeated ISO (25mg/kg over 5 consecutive days) administration to mice induced a significant decrease in LV MMP-13 (collagenase-3; **a,b**), MMP-9 (gelatinase B; **a,c**) and MMP-2 (gelatinase A; **a,d**), but a significant increase in LV TIMP-1 (**a,e**) and TIMP-2 (**a,f**) expression levels at day-14 post-injury. This corresponded to a significant decrease in LV MMP-13/TIMP-1 ratio (**g**), MMP-9/TIMP-1 ratio (**h**) and MMP-2/TIMP-2 ratio (**i**) at day-14 post-injury. (**a**) Representative Western blots (of MMP-13, TIMP-1, TIMP-2) or gelatin zymographs (of MMP-9, MMP-2) show the LV expression levels of each MMP or TIMP evaluated in each of the groups investigated. Also shown is the relative mean $\pm$ standard error of the mean (SEM) optical density (OD) of LV MMP-13 (**b**), MMP-9 (**c**), MMP-2 (**d**), TIMP-1 (**e**), TIMP-2 (**f**), MMP-13/TIMP-1 ratio (**g**), MMP-9/TIMP-1 ratio (**h**) and MMP-2/TIMP-2 ratio (**i**) in each the groups evaluated; where the data were acquired from densitometry measurements of the corresponding bands identified in the Western blots or gelatin zymographs, and expressed as a relative value to the saline (SAL) control group (which was expressed as 1 in each case). The intraperitoneal (i.p) administration of SPION-RLX (25ng/day; on days 7, 10 and 13 post-ISO-injury) or daily drinking water (p.o) administration of SPION-RLX (25ng/day; from days 7-14 post-ISO-injury) was able to stimulate an increase in LV MMP-9 (**c**) and MMP-2 (**d**) expression levels, as well as the MMP-9/TIMP-1 (**h**) and MMP-2/TIMP-2 ratio (**i**); restore the ISO-induced loss of LV MMP-13/TIMP-1 ratio (back to that detected in saline-injected control mice (**g**)); and reduce LV TIMP-1 (**e**) and TIMP-2 (**f**) expression levels to a similar or trend towards an improved extent over Pump-infused RLX (0.5mg/kg/day; from days 7-14 post-ISO-injury), at day 14-post-injury. However, the Empty-SPIONs alone were not able to achieve any of the effects of i.p-administered or p.o-administered SPION-RLX. The data presented in panels **b-i** were obtained from n=7-8 mice per group. The white coloured circles in each of the bar plots represent the individual data points per group. *P<0.05, **P<0.01 versus the saline group;

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[#]P<0.05, ^{##}P<0.01 versus the ISO group; [†]P<0.05, ^{††}P<0.01 versus the ISO+Empty-SPION-treated group; as determined by a non-parametric Kruskal Wallis test and Dunn's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0023] **Figure 11. The anti-fibrotic effects of orally (p.o)-administered SPION-RLX or SPION-B7-33 versus the ACE inhibitor, Perindopril, versus unconjugated RLX or B7-33 in mice with ISO-induced cardiomyopathy, when delivered from days 14-42 post-ISO injury.** Repeated ISO (25mg/kg over 5 consecutive days) administration to mice induced a significant increase in interstitial LV fibrosis within the LV at 42-days post-injury, compared to respective measurements obtained from saline-treated control mice. (a) Representative images (with enlarged insets) of picrosirius red-stained LV tissue sections, show the extent of interstitial collagen (red) deposition within the LV midzone in each of the groups investigated. (b) Also shown is the mean $\pm$ standard error of the mean (SEM) % interstitial LV collagen deposition (fibrosis) per field (as determined by morphometric analysis of 8-10 random and non-overlapping fields of view per section). This ISO-induced increase in interstitial LV fibrosis was abrogated by the oral (p.o) administration of SPION-RLX (25ng/day) or SPION-B7-33 (25ng/day), partially but significantly reduced by p.o-administered Perindopril treatment (60ng/day) but unaffected by p.o-administered unconjugated RLX (25ng/day) or B7-33 (25ng/day) or Empty-SPIONs alone, when given every 3 days from days 14-42 (4 week period) post-ISO injury. The data presented in panels b was obtained from n=6-7 mice per group. The white coloured circles in each of the bar plots represent the individual data points per group. ^{**}P<0.01, ^{***}P<0.001 versus the saline group; [#]P<0.05, ^{##}P<0.001 versus the ISO group; ^{†††}P<0.001 versus the ISO+Empty-SPION-treated group; ^{§§§}P<0.001 versus the ISO+(unconjugated) RLX-treated group; ^{†††}P<0.001 versus the ISO+(unconjugated) B7-33-treated group; [‡]P<0.05 versus the ISO+Perindopril-treated group; as determined by a one-way ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0024] **Figure 12. The anti-hypertrophic effects of orally (p.o)-administered SPION-RLX or SPION-B7-33 versus the ACE inhibitor, Perindopril, versus unconjugated RLX or B7-33 in mice with ISO-induced cardiomyopathy, when delivered from days 14-42 post-ISO injury.** Repeated ISO (25mg/kg over 5 consecutive days) administration to mice induced a significant increase in LV cardiomyocyte hypertrophy (enlargement) at 42-

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days post-injury, compared to respective measurements obtained from saline-treated control mice. (a) Representative images of picrosirius red-stained LV tissue sections, show the extent of LV cardiomyocyte size in each of the groups investigated. (b) Also shown is the relative mean $\pm$ standard error of the mean (SEM) LV cardiomyocyte cross sectional area in each of the groups analysed (which was determined from 100 cardiomyocytes in 8-10 random and non-overlapping fields of view per section). This ISO-induced increase in interstitial LV fibrosis was abrogated by the oral (p.o) administration of SPION-RLX (25ng/day), SPION-B7-33 (25ng/day) or Perindopril treatment (60ng/day), but unaffected by p.o-administered unconjugated RLX (25ng/day) or B7-33 (25ng/day) or Empty-SPIONs alone, when given every 3 days from days 14-42 (4 week period) post-ISO injury. The data presented in panel b was obtained from n=6-7 mice per group. The white coloured circles in each of the bar plots represent the individual data points per group. ***P<0.001 versus the saline group; #P<0.05 versus the ISO group; †P<0.05 versus the ISO+Empty-SPION-treated group; §P<0.05 versus the ISO+(unconjugated) RLX-treated group; †P<0.05 versus the ISO+(unconjugated) B7-33-treated group; as determined by a one-way ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0025] **Figure 13. The angiogenic effects of orally (p.o)-administered SPION-RLX or SPION-B7-33 versus the ACE inhibitor, Perindopril, versus unconjugated RLX or B7-33 in mice with ISO-induced cardiomyopathy, when delivered from days 14-42 post-ISO injury.** Repeated ISO (25mg/kg over 5 consecutive days) administration to mice induced a significant loss of smooth muscle actin-stained blood vessel density at 42-days post-injury, compared to respective measurements obtained from saline-treated control mice. (a) Representative immunohistochemically-stained sections for α -smooth muscle actin (SMA), show the extent of α -SMA-stained blood vessel density in each of the groups investigated. (b) Also shown is the mean $\pm$ SEM LV blood vessel density per group analysed (from the morphometric analysis of 8-10 random and non-overlapping fields of view per). This ISO-induced increase in interstitial LV fibrosis was abrogated by the oral (p.o) administration of SPION-RLX (25ng/day) or SPION-B7-33 (25ng/day) but not by p.o-administered Perindopril (60ng/day), unconjugated RLX (25ng/day), unconjugated B7-33 (25ng/day) or Empty-SPIONs alone treatment, when given every 3 days from days 14-42 (4 week period) post-ISO injury. The data presented in panel b was obtained from n=6-7 mice per group. The white coloured circles in each of the bar plots represent the individual

data points per group. ***P<0.001 versus the saline group; ###P<0.001 versus the ISO group; ****P<0.001 versus the ISO+Empty-SPION-treated group; §§§P<0.001 versus the ISO+(unconjugated) RLX-treated group; †††P<0.001 versus the ISO+(unconjugated) B7-33-treated group; †††P<0.001 vs the ISO+Perindopril-treated group; as determined by a one-way ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

[0026] **Figure 14. Schematic overview of how mice were induced to undergo interstitial lung fibrosis over a 4-week period and treated with minipump (Pump)-AT2R agonist versus oral gavage (p.o)-administered AT2R agonist versus p.o-administered SPION-AT2R agonist from days 21-28 post-BLM injury.** 11-12-week-old male C57BL/6 mice were intranasally administered on days 0 and day 7 with either i) saline (vehicle for bleomycin (BLM); non-injury control group; n=40) or ii) BLM (0.15mg/day; injury control group; n=8). The saline-administered group (n=8) and one sub-group of BLM-injured mice (n=8) was left untreated for a further 21 days, until day 28. The other subgroups of BLM injured mice were subjected to either iii) subcutaneous minipump implantation of 0.1mg/kg/day of the AT2R agonist, beta-Pro⁷-Trp⁸-angiotensin III, on day 21 post-BLM-induced injury (which continuously infused the AT2R agonist into the circulation of treated mice for a 7 day period; from days 21-28; positive treatment control; BLM+Pump-AT2R agonist group; n=8); iv) 17.5µg/day of the AT2R agonist (beta-Pro⁷-Trp⁸-angiotensin III) alone through oral gavage every 72 hours (on days 21, 24 and 27; BLM+AT2R agonist (p.o) group; n=8); v) 17.5µg/day SPION-AT2R agonist (SPION- beta-Pro⁷-Trp⁸-angiotensin III) through oral gavage every 72 hours (on days 21, 24 and 27; BLM+SPION-AT2R agonist (p.o) group; n=8); or vi) empty SPIONs that were not conjugated to AT2R agonist through oral gavage every 72 hours (on days 21, 24 and 27; BLM+Empty-SPION vehicle control (p.o) group; n=8). All mice were killed for tissue collection and analysis on day 28.

[0027] **Figure 15. The effects of minipump (Pump)-AT2R agonist versus drinking water (p.o)-administered AT2R agonist versus p.o-administered SPION-AT2R agonist on interstitial lung fibrosis in mice with BLM-induced interstitial lung fibrosis, when delivered from days 21-28 post-BLM injury.** Intranasal administration of BLM (0.15mg/day on days 0 and 7) to mice induced a 2.5-fold increase in picrosirius red-stained interstitial lung fibrosis at day 28 post-injury. (a) Representative images of picrosirius red-

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stained interstitial lung tissue sections show the extent of fibrosis (as visualised by the number of red-stained interstitial lung collagen deposition) in each of the groups investigated. (b) Interstitial lung fibrosis by % fractional area for each of the groups indicated. Also shown are the mean $\pm$ standard error of the mean (SEM) number of interstitial lung fibrosis staining per field (as determined by morphometric analysis of 10 random and non-overlapping fields of view per section). The oral gavage (p.o) administration of SPION-AT2R agonist (17.5 μ g/day; on days 21, 24 and 27 post-BLM-induced injury) was able to reduce the BLM-induced increase in fibrosis to the same extent as Pump-infused AT2R agonist (0.1mg/kg/day; from days 21-28 post-BLM-injury), at day 28-post-injury. However, these anti-fibrotic effects of the p.o-administered SPION-AT2R agonist were not achieved by p.o-administration of the AT2R agonist alone (that was not conjugated to SPIONs) or Empty SPIONs alone. The white coloured circles in each of the bar plots represent the individual data points per group. *P<0.05, **P<0.01, ***P<0.001 versus the saline group; #P<0.05, ##P<0.01, ###P<0.001 versus the BLM group; ¶P<0.05, ¶¶P<0.01, ¶¶¶P<0.001 versus the BLM+Empty-SPION-treated group; as determined by a one-way ANOVA and Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown.

GENERAL DEFINITIONS

[0028] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

[0029] Unless otherwise specified, the indefinite articles “a”, “an” and “the” as used herein, include plural aspects. Thus, for example, reference to “an agent” includes a single agent, as well as two or more agents; reference to “the composition” or “the formulation” includes a single composition or formulation, as well as two or more compositions or formulations; and so forth.

[0030] As used herein, and unless the context indicates otherwise, the term “about” means $\pm 10\%$ of the recited value.

[0031] Throughout this specification and the claims that follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will

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be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

[0032] The term “consisting of” means “consisting only of”, that is, including and limited to the integer or step or group of integers or steps, and excluding any other integer or step or group of integers or steps.

[0033] The term “consisting essentially of” means the inclusion of the stated integer or step or group of integers or steps, but other integer or step or group of integers or steps that do not materially alter or contribute to the working of the invention may also be included.

[0034] In the context of the present specification, the terms “composition” and “formulation” are used interchangeably and are intended to have the same meaning.

[0035] The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge.

[0036] Other definitions may be found throughout the description.

DETAILED DESCRIPTION

[0037] The present invention broadly relates to oral delivery of peptides. In particular, the present invention relates to conjugates comprising a peptide and a superparamagnetic iron oxide nanoparticle (SPION), which may be suitable for orally delivering the peptide and may advantageously provide improved oral bioavailability of the peptide. The present invention also relates to oral dosage forms comprising the SPION-peptide conjugates described herein, which may be suitable for delivering a variety of peptides for therapeutic or diagnostic purposes. Thus, oral delivery of peptides using the SPION-peptide conjugates disclosed herein may provide an alternative or improved mode of delivery of peptides compared to other forms of delivery.

[0038] In particular, the present invention provides a method of orally delivering a peptide to a subject, the method comprising conjugating the peptide to a glycine-coated SPION to provide a SPION-peptide conjugate, and orally delivering the SPION-peptide conjugate to

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the subject. The present invention further provides use of a glycine-coated SPION conjugated to a peptide for orally delivering the peptide to a subject.

[0039] Super-paramagnetic iron oxide nanoparticles (SPIONs; also referred to herein as nanoparticles (NPs)) are synthetic nanoparticles that are non-cytotoxic, biocompatible, and biodegrade by about 48-72 hours post-administration. Thus, SPIONs may be particularly suitable for orally delivering a peptide to a subject, as they may reduce or prevent the breakdown of the peptide in the gastrointestinal tract, allowing the peptide to be absorbed systemically and delivered to the site of action. Without wishing to be bound by theory or a particular mode of action, the present inventors postulate that GSPION-peptide conjugates as disclosed herein are taken up by myeloid dendritic cells and/or macrophages in the gut, protecting the peptides from degradation. Further, the glycine coating on the SPIONs may act as a buffer to protect the peptides from degradation upon exposure to the acidic gut environment.

[0040] SPIONs exhibit magnetic properties in the presence of an external magnetic field. This property of superparamagnetism may allow for targeted delivery of therapeutic or diagnostic agents to various sites within the body by applying a magnetic field (e.g., using Magnetic Drug Targeting, Magnetic Hyperthermia, or Magnetic Resonance Imaging). When the magnetic field is removed, SPIONs are easily dispersed, evading uptake by phagocytes and leading to a longer half-life in the circulation. SPIONs typically comprise γ -Fe₂O₃ (maghemite), Fe₃O₄ (magnetite) or α -Fe₂O₃ (hermatite), but can also include mixed oxides of iron with transition metal ions such as copper, cobalt, nickel, and manganese. In particular, spherical magnetite and maghemite nanoparticles may provide a uniform surface area for coating and conjugation of targeting ligands or therapeutic agents. Thus, in an embodiment, SPIONs suitable for use in the present invention comprise magnetite nanoparticles, maghemite nanoparticles, or a combination thereof.

[0041] When used without modification, SPIONs can cause unwanted toxicity and inflammation in the body. Thus, SPIONs suitable for use in the present invention may be coated with glycine, preferably D-glycine. Advantageously, coating the SPIONs with glycine may: improve the affinity and stability of the conjugation of peptides to SPIONs; diminish the immune response and subsequent pro-inflammatory reaction evoked in the host post-administration compared to other nanoparticle-drug conjugates; prevent build-up of

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SPIONs in tissues that would otherwise lead to chronic inflammation; and/or allow for the uptake of SPION-peptide conjugates by infiltrating and resident immune cells to the target site, which enables the SPIONs to release the peptides in a targeted manner. Without wishing to be bound by theory, it is postulated that the glycine is chemisorbed onto the surface of the SPION to provide free carboxyl groups on the surface of the SPION. Suitable processes for coating SPIONs with glycine may include a modified alkaline co-precipitation method, such as the method described by Barick and Hassan, 2012. Such methods may provide an average of about 20.2×10^3 glycine molecules conjugated to each SPION particle.

[0042] Further, peptides may be conjugated to glycine-coated SPIONs (GSPIONs) using any suitable method known in the art. For example, the N-terminus of the peptide may be covalently coupled to the free carboxyl group of the glycine on the surface of the SPION via an amide bond. Suitable methods for forming the amide bond may include a carbodiimide reaction, for example, according to the method described by Chakraborty et al., 2021. For example, peptide conjugation using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) involves reacting an exposed glycine carboxyl group on the surface of the SPION with the EDC to form an ester. This ester intermediate is then attacked by a nucleophile, being the N-terminus of the relevant peptide. The resultant SPION-peptide conjugates may comprise a molar ratio of GSPION to peptide from about 4:1 to about 20:1. For example, the molar ratio of glycine-coated SPION to peptide may be about 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, or 20:1. The molar ratio of GSPION to peptide may vary depending on the size of the peptide and steric hindrance provided by the peptide upon attachment at the surface of the GSPION. Typically, GSPION-peptide conjugates suitable for use in the present invention may be prepared by reacting GSPION at a concentration from about 200 µg/mL to about 400 µg/mL with a peptide at a concentration about 10 µg/mL to about 50 µg/mL.

[0043] Advantageously, conjugating a peptide to a GSPION may improve the oral bioavailability of the peptide. Without wishing to be bound by theory, it is postulated that the GSPION-peptide conjugates described herein are taken up by myeloid dendritic cells and absorbed through draining lymph nodes into the circulation, where they biodegrade to release the peptide. As used herein, the term “oral bioavailability” with reference to a peptide means the extent to which the peptide is systemically available (e.g., in blood plasma) when

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administered orally. Improved oral bioavailability of a peptide when administered orally as a GSPION-peptide conjugate as disclosed herein may include enhanced gut absorption of the peptide, decreased metabolism of the peptide in the gut, decreased decomposition of the peptide in the gut, or decreased efflux of the peptide in the gut, or a combination thereof, relative to the unconjugated peptide. A skilled person will be readily able to measure an improvement in oral bioavailability of a peptide by comparing the plasma concentration of the peptide after administration of a GSPION-peptide conjugate to the plasma concentration of the unconjugated peptide. Suitable methods of measuring the plasma concentration of a peptide will be apparent to those skilled in the art and may include, for example, an Enzyme-linked immunosorbent assay (ELISA) assay.

[0044] In accordance with the present invention, GSPIONs may be conjugated to any suitable peptide, or an analogue thereof, for oral delivery, particularly peptides that are poorly absorbed in the gut. The term “peptide” as used herein refers to any molecule of two or more amino acids or analogues thereof linked through peptide bonds, modified peptide bonds, or other suitable bonds (e.g., ester bonds, ether bonds, and the like). Peptides suitable for use in the present invention may include amino acid polymers in which one or more amino acid residues is a non-naturally occurring (synthetic) amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers. Thus, the term “peptide” as used herein may encompass dipeptides, polypeptides, proteins, antibodies, and analogues thereof. As used here, a peptide “analogue” refers to a naturally occurring (native) peptide the sequence of which has been adapted to improve the therapeutic potential of the peptide, for example, by modification of the amino acid sequence or through conjugation with other molecules. In an embodiment, the peptide analogue may have an amino acid sequence having at least 80% sequence identity to the amino acid sequence of the native peptide, e.g., 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the native peptide sequence after optimal alignment or best fit analysis.

[0045] Peptides suitable for use in the present invention may include, but are not limited to, naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Peptides suitable for use

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in the present invention may comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a peptide by the cell in which the peptide is produced, and will vary with the type of cell. For peptides that are made recombinantly, the nature and extent of the modifications in large part will be determined by the post-translational modification capacity of the particular host cell and the modification signals that are present in the amino acid sequence of the peptide in question. For instance, glycosylation patterns vary between different types of host cell. Peptides are defined herein, in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless. In addition, peptides suitable for use in the present invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Proteins may be present as monomeric or as multimeric proteins e.g., as dimers (homo or heterodimers) or trimers.

[0046] In the context of this specification, the term “amino acid” is defined as having at least one primary, secondary, tertiary or quaternary amino group, and at least one acid group, wherein the acid group may be a carboxylic, sulfonic, or phosphonic acid, or mixtures thereof. Preferably, the acid group is a carboxylic acid group. The amino groups may be “alpha”, “beta”, “gamma” ... to “omega” with respect to the acid group(s). The backbone of the “amino acid” may be substituted with one or more groups selected from halogen, hydroxy, guanido, heterocyclic groups. Thus term “amino acids” also includes within its scope glycine, alanine, valine, leucine, isoleucine, methionine, proline, phenylalanine, tryptophane, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamine, lysine, arginine and histidine, taurine, betaine, N-methylalanine etc. (L) and (D) forms of amino acids are included in the scope of this invention.

[0047] Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions consist of replacing one or more amino acids of the peptide sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics, such as, for example, a glutamic acid (E) to aspartic acid (D) amino acid substitution. When conserved substitutions are made, the resulting peptide may be functionally equivalent to peptide from which it is derived. Non-conserved substitutions consist of replacing one or more amino acids of the peptide sequence with amino acids possessing dissimilar charge,

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size, and/or hydrophobicity characteristics, such as, for example, a glutamic acid (E) to valine (V) substitution.

[0048] Amino acid insertions may consist of single amino acid residues or stretches of residues ranging from 2 to 15 amino acids in length. One or more insertions may be introduced into the peptide sequence. The insertions may be such that the amino acid sequence is still conserved. The insertion may be such that a part (or the whole) of the amino acid sequence is repeated. The insertions may occur at the ends of the amino acid sequence or inserted within the sequence.

[0049] Deletions of amino acids in the peptide sequence are also within the scope of the invention. Such deletions consist of the removal of one or more amino acids from the peptide sequence, with the lower limit length of the resulting peptide sequence being 4 to 6 amino acids. Such deletions may involve a single contiguous or greater than one discrete portion of the peptide sequences.

[0050] The peptides suitable for use in the present invention may be synthesized or prepared by techniques well known in the art. See, for example, Creighton, 1983, *Proteins: Structures and Molecular Principles*, W. H. Freeman and Co., N.Y., which is incorporated herein by reference in its entirety. Short peptides, for example, can be synthesized on a solid support or in solution. Longer peptides may be made using recombinant DNA techniques. Here, the nucleotide sequences encoding the peptides of the invention may be synthesized, and/or cloned, and expressed according to techniques well known to those of ordinary skill in the art. See, for example, Sambrook, et al., 2001, *Molecular Cloning: A Laboratory Manual*.

[0051] The peptides suitable for use in the present invention may alternatively be synthesized such that one or more of the bonds which link the amino acid residues of the peptides are non-peptide bonds. These alternative non-peptide bonds may be formed by utilizing reactions well known to those in the art, and may include, but are not limited to imino, ester, hydrazide, semicarbazide, and azo bonds, to name but a few. In an embodiment, peptides comprising the sequences described above may be synthesized with additional chemical groups present at their amino and/or carboxy termini, such that, for example, the stability, bioavailability, and/or inhibitory activity of the peptides is enhanced. For example, hydrophobic groups such as carbobenzoxy, dansyl, or t-butyloxycarbonyl groups, may be

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added to the peptides' amino termini. Likewise, an acetyl group or a 9-fluorenylmethoxycarbonyl group may be placed at the peptides' amino termini. Additionally, the hydrophobic group, t-butyloxycarbonyl, or an amido group may be added to the peptides' carboxy termini. Further, peptides suitable for use in the present invention may be synthesized such that their steric configuration is altered. For example, the D-isomer of one or more of the amino acid residues of the peptide may be used, rather than the L-isomer. Still further, at least one of the amino acid residues of peptides suitable for use in the present invention may be substituted by one of the well known non-naturally occurring amino acid residues. Alterations such as these may serve to increase the stability, bioavailability and/or inhibitory action of the peptides of the invention.

[0052] At least one amino acid residue of the peptide suitable for use in the present invention may be substituted by unnatural amino acid residues and their derivatives. Unnatural amino acids and derivatives can be, but is not limited to, β -amino acids, homo-amino acids, proline and pyruvic acid derivatives, 3-substituted alanine derivatives, glycerine derivatives, ring-substituted phenylalanine and tyrosine derivatives, linear core amino acids and N-methyl amino acids. Other derivatives of amino acids can be, but is not limited to, 2-amino adipic acid (Aad) for Glutamic acid and Aspartic acid; 2-aminopimelic acid (Apm) for Glutamic acid and Aspartic acid; 2-aminobutyric (Abv) acid for Methionine, Leucine, and other aliphatic amino acids; 2-aminoheptanoic acid (Ahe) for Methionine, Leucine and other aliphatic amino acids; 2-aminoisobutyric acid (Alb) for Glycine; cyclohexylalanine (Cha) for Valine, Leucine and Isoleucine; homoarginine (Har) for Arginine and Lysine; 2,3-diaminopropionic acid (Dpr) for Lysine, Arginine and Histidine; N-ethylglycine (EtGly) for Glycine, Proline, and Alanine; N-ethylglycine (EtGly) for Glycine, Proline, and Alanine; N-ethylasparagine (EtAsn) for Asparagine, and Glutamine; hydroxyllysine (Hyl) for Lysine; alcohoxyllysine (AHyl) for Lysine; 3-hydroxyproline and 4-hydroxyproline (3Hyp, 4Hyp) for Proline, Serine, and Threonine; allo-isoleucine (Alle) for Isoleucine, Leucine, and Valine; p-amidinophenylalanine for Alanine; N-methylglycine (MeGly, sarcosine) for Glycine, Proline, and Alanine; N-methylisoleucine (Merle) for Isoleucine; Norvaline (Nva) for Methionine and other aliphatic amino acids; Norleucine (Nle) for Leucine and other aliphatic amino acids; Ornithine (Orn) for Lysine, Arginine and Histidine; Citrulline (Cit) and methionine sulfoxide (MSO) for Threonine, Aspartic acid and Glutamic

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acid; methylphenylalanine (MePhe), trimethylphenylalanine, halo (F, Cl, Br, and I)phenylalanine, trifluoromethylphenylalanine, for Phenylalanine.

[0053] Peptides suitable for use in the present invention may include therapeutic peptides, diagnostic peptides, and analogues thereof. Suitable therapeutic or diagnostic peptides for use in the present invention will be apparent to those skilled in the art and may be selected based on the disease or condition to be treated or diagnosed. It will be apparent from the context in which the word “peptide” appears whether it includes, therapeutic peptides, diagnostic peptides, or a combination thereof.

[0054] The particle size of a GSPION-peptide conjugate may determine its half-life in the circulation. Smaller sized particles (e.g., less than 10 nm) may be removed from circulation by renal clearance and/or diffuse through cell membranes causing damage to organelles, whereas larger particle sizes (e.g., greater than 200 nm) may become concentrated in the spleen or may be taken up by phagocytic cells, thereby reducing plasma concentrations. The GSPION-peptide conjugates for use in the present invention preferably have a hydrodynamic diameter (D_h) from about 20 nm to about 100 nm, e.g., about 20 nm to about 100 nm, e.g., about 20 nm, about 30 nm, about 40 nm, about 50 nm, about 60 nm, about 70 nm, about 80 nm, about 90 nm, or about 100 nm. To achieve a suitable hydrodynamic diameter, the GSPIONs are preferably conjugated to peptides having an atomic mass of about 170 kDa or less. For example, the peptides may have atomic mass from about 0.1 kDa to about 170 kDa, from about 1 kDa to about 170 kDa, from about 3 kDa to about 170 kDa, from about 5 kDa to about 170 kDa, from about 5 kDa to about 150 kDa, from about 5 kDa to about 100 kDa, or from about 5 kDa to about 100 kDa. Further, the GSPION core preferably has a hydrodynamic diameter (D_h) of 20 nm or less, e.g., from about 1 to about 20 nm, or about 2 nm to about 15 nm, or about 5 nm to about 12 nm. As used herein, the term “hydrodynamic diameter (D_h)” refers to the diameter of a perfect solid sphere formed with a solvent layer *in vivo* (in a biological system) that would exhibit the same hydrodynamic friction as the GSPION-peptide conjugate of interest in a biological system. The particle size (or hydrodynamic diameter) of the GSPION-peptide conjugates (or GSPION core) may be measured, for example, using transmission electron microscopy (TEM), dynamic light scattering, or the Scherrer method using x-ray diffractograms. If necessary, GSPION-peptide conjugates of the desired size (e.g., 20 nm to 100 nm) may be

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separated from conjugates of undesirable size, for example, using centrifugation, size exclusion chromatography, or field flow fractionation.

[0055] The GSPION-peptide conjugates disclosed herein may be formulated for oral delivery. Thus, in an embodiment, the present invention provides oral dosage forms comprising a glycine-coated SPION conjugated to a peptide (i.e., a GSPION-peptide conjugate). By way of non-limiting example, the oral dosage form may be a tablet, capsule, gelcap, caplet, chewable tablet, effervescent tablet, lozenge, dispersible powder, granule, syrup, elixir, solution or suspension in aqueous or non-aqueous liquid, edible foam or whip, oil-in-water liquid emulsion or water-in-oil liquid emulsion, and the like. In an embodiment, the oral dosage form is a tablet, capsule or gelcap.

[0056] The SPION-peptide conjugates disclosed herein may be provided as pharmaceutical compositions comprising the SPION-peptide conjugate and at least one pharmaceutically acceptable excipient (e.g. carriers, diluents, etc.). It is also contemplated that the SPION-peptide conjugates disclosed herein may be suitable for use in veterinary applications. Thus term “pharmaceutically acceptable excipient” is also intended to include veterinarily acceptable excipients. Where an excipient is used, it must be “pharmaceutically acceptable” in the sense of being compatible with the other ingredients of the pharmaceutical formulation and not injurious to the subject. Pharmaceutically acceptable excipients suitable for oral administration will be apparent to those skilled in the art and may depend on the intended oral dosage form (e.g., tablet, capsule, gelcap, etc.). Examples of suitable pharmaceutically acceptable excipients for use in the pharmaceutical compositions disclosed herein may include diluents, binders, disintegrants, lubricants, glidants, emulsifiers, and the like. In some embodiments, the pharmaceutically acceptable excipient may be an auxiliary substances as required to approximate physiological conditions, such as pH-adjusting and buffering agents, tonicity-adjusting agents, wetting agents and the like, for example sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, tris(hydroxymethyl)aminomethane (Tris), sorbitan monolaurate, triethanolamine oleate, sucrose or other carbohydrates, among many others. The pharmaceutical compositions used in the present invention may be sustained-release formulations for oral delivery.

[0057] Suitable diluents for use in the present invention may include, but are not limited to, starch, microcrystalline cellulose, dicalcium phosphate, lactose, sorbitol, mannitol, sucrose,

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dextrose, glycine, methyl dextrans, and any combination thereof. Suitable binders for use in the present invention may include, but are not limited to, povidone, hydroxypropyl methylcellulose, dihydroxy propyl cellulose, sodium carboxymethylcellulose magnesium aluminum silicate, starch paste, gelatin, tragacanth, methylcellulose, polyvinylpyrrolidone, acacia, and any combination thereof. Suitable disintegrants for use in the present invention may include, but are not limited to, crospovidone, sodium starch glycolate, croscarmellose sodium, agar, alginic acid or its sodium salt, and any combination thereof. Suitable lubricants for use in the present invention may include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, glyceryl behenate, hydrogenated vegetable oil, glycerine fumarate, silica, talc, polyethyleneglycol, and any combination thereof. Suitable glidants for use in the present invention may include, but are not limited to, colloidal silicon dioxide. In some embodiments, the pharmaceutically acceptable excipient may be selected from microcrystalline cellulose, starch, talc, povidone, crospovidone, magnesium stearate, colloidal silicon dioxide, sodium dodecyl sulfate, and any combination thereof. The excipients may be intragranular, intergranular, or mixtures thereof.

[0058] In some embodiments, the pharmaceutical compositions disclosed herein may be prepared as solid formulations, including freeze-dried formulations. In other embodiments, the pharmaceutical compositions disclosed herein may be prepared as liquid formulations. Representative pharmaceutically acceptable carriers include any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except, insofar as any conventional carrier is incompatible with the active ingredient(s), its use in the pharmaceutical compositions is contemplated. Liquid formulations may include pharmaceutically acceptable solutions, syrups, slurries, emulsions, microemulsions, suspensions or other multiphasic compositions or dispersions. Suitable liquid carriers may include any suitable organic or non-organic solvent, for example, water, alcohol, saline solution, buffered saline solution, physiological saline solution, dextrose solution, water propylene glycol solutions, and the like, preferably in

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sterile form. Other common liquid formulations may include sodium phosphate solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives can also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. After formulation, the pharmaceutical composition may be incorporated into a sterile container, which is then sealed and stored at a suitable temperature.

[0059] Tablets suitable for use in the present invention may be coated or uncoated. For example, tablets may be film coated or enteric coated according to methods known in the art. The tablets may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed. Capsules or gelcaps suitable for use in the present invention may be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin or olive oil.

[0060] The pharmaceutical composition may comprise the SPION-peptide conjugate in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active peptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[0061] Various other pharmaceutical compositions suitable for oral administration and methods of preparing pharmaceutical compositions will be known in the art, illustrative examples of which are described in "Remington: The Science and Practice of Pharmacy" (formerly "Remingtons Pharmaceutical Sciences"); Gennaro, A., Lippincott, Williams & Wilkins, Philadelphia, Pa. (2000). In general, such preparatory methods include the steps of bringing the SPION-peptide conjugate into association with one or more excipients and then,

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if necessary and/or desirable, shaping and/or packaging the product into a desired single- or multi-dose unit.

[0062] In certain embodiments, unit dosage compositions are those containing a daily dose or unit, daily sub-dose, as herein above described, or an appropriate fraction thereof, of the SPION-peptide conjugate. As used herein, a “unit dose” is a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient (i.e., the SPION-peptide conjugate) that would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[0063] The present inventors found that conjugating a peptide to a SPION, particularly a glycine-coated SPION (GSPION), allows for systemic delivery of the peptide via oral administration. Further, targeted delivery of the SPION-peptide conjugate may be achieved, for example, by conjugating cell specific receptors or other antigenic target specific immunoglobulins with their Fc region. Accordingly, GSPION-peptide conjugates may be suitable for treating or diagnosing diseases or conditions the peptide is known to treat or diagnose, or that will in future be identified as treating or diagnosing, by orally delivering the GSPION-peptide conjugate to the subject.

[0064] Thus, the present invention provides a method of treating a disease or condition in a subject, the method comprising orally administering to the subject a glycine-coated SPION conjugated to a therapeutic peptide, wherein the disease or condition is treatable with the peptide.

[0065] The present invention also provides a method of diagnosing a disease or condition in a subject, the method comprising orally administering to the subject a glycine-coated SPION conjugated to a diagnostic peptide, wherein the disease or condition is diagnosable with the peptide.

[0066] The present invention further provides use of a glycine-coated SPION conjugated to a therapeutic peptide, in the manufacture of a medicament for treating a disease or condition in a subject by oral administration, wherein the disease or condition is treatable with the

therapeutic peptide.

[0067] The present invention further still provides a glycine-coated SPION conjugated to a peptide for use in treating or diagnosing a disease or condition in a subject by oral administration, wherein the disease or condition is treatable or diagnosable with the peptide.

[0068] Illustrative therapeutic or diagnostic peptides (and the indications they may be suitable to treat) may include, but are not limited to: relaxin peptide receptor (RXFP1) agonists such as serelaxin and B7-33 (heart failure); angiotensin type 2 receptor (AT2R) agonists such as angiotensin II (hypotension resulting from septic shock or other distributive shock), angiotensin III (hypotension resulting from septic shock or other distributive shock), angiotensin 1-7 (heart failure and cardiac hypertrophy), CGP42112 (full AT2R agonist effectively used in targeting the RAAS) and beta-Pro⁷-angiotensin III (hypertension); GLP-1 receptor agonists such as exenatide, liraglutide, lixisenatide, albiglutide, dulaglutide and semaglutide agonists (type 2 diabetes mellitus); GLP-1 receptor antagonists such as avexitide (hypoglycaemia); GLP-2 analogues such as teduglutide (short bowel syndrome and malabsorption); GC-C receptor agonists such as linaclotide (irritable bowel syndrome (IBS) with constipation and chronic idiopathic constipation); calcitonin receptor agonists such as pramlintide (type 1 and type 2 diabetes mellitus); GnRH receptor antagonists such as abarelix and degarelix (advanced prostate cancer); proteasome inhibitors such as carfilzomib, bortezomib and ixazomib (multiple myeloma); NOD2 protein agonists such as mifamurtide (high-grade, resectable, non-metastatic osteosarcoma); vasoactive intestinal peptides (VIP) such as aviptadil (erectile dysfunction); OT receptor antagonists such as atosiban (delaying imminent pre-term birth) and carbetocin (postpartum haemorrhage); TRH analogues such as taltirelin (spinocerebellar degeneration); MC receptor agonists such as bremelanotide (hypoactive sexual desire disorder); PTH1 receptor agonists such as teriparatide and abaloparatide (osteoporosis); guanylate cyclase C agonists such as plecanatide (chronic idiopathic constipation); NPR-A agonists such as nesiritide (acute decompensated heart failure); β 2-receptor antagonists such as icatibant (acute attacks of hereditary angioedema); gp41 inhibitors such as enfuvirtide (combination therapy for the treatment of HIV-1); GHRH analogues such as tesamorelin (reduction of HIV lipodystrophy); N-type calcium channel antagonists such as ziconotide (management of severe chronic pain); thrombopoietin receptor agonists such as romiplostim and eltrombopag

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(chronic immune thrombocytopenic purpura); human erythropoietin receptor agonists such as peginesatide (anemia associated with chronic kidney disease); pulmonary surfactants such as lucinactant (respiratory distress syndrome); CaSR agonists such as etelcalcetide (secondary hyperparathyroidism); MC1 receptor agonists such as afamelanotide (phototoxicity) and pasireotide (Cushing's disease); somatostatin analogues such as lutetium Lu 177 dotatate (somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors) and edotreotide gallium Ga-68 (diagnosis of somatostatin receptor positive neuroendocrine tumors); melanocortin-4 receptor agonists such as setmelanotide (chronic weight management of obesity); farnesoid X receptor (FXR) agonists such GLP-1 (chronic liver diseases); Peptide DR8 analogues such as DR8-3D and DR8-8A (pulmonary fibrosis).

[0069] Suitable therapeutic and diagnostic peptides may be readily selected by a person skilled in the art pending on the disease or condition to be treated or diagnosed. In the case of diagnostic peptides, the peptide may be tagged, for example, by fluorescence labelling. Suitable methods of fluorescence labelling will be apparent to those skilled in the art and may include, for example, Fluorescein isothiocyanate (FITC) labelling. Such FITC labelled peptides may be used for identifying the cells where the reference peptide interacts using fluorescence activated cell sorting (FACS) analysis.

[0070] By way of non-limiting example, peptide-based therapeutics have great potential as anti-fibrotic agents. Fibrosis (tissue scarring) is associated with many chronic inflammatory diseases and contributes to around 45% of all deaths in developed countries. Fibrosis results from a failed or maladaptive wound-healing response to tissue injury, and is characterised by the prodigious build-up of various excessive extracellular matrix (ECM) proteins, primarily collagen. Upon chronic or repetitive injury to various organs, the ECM that forms to offer structural support during tissue repair, uncontrollably accumulates, leading to the formation of a pathological fibrotic scar which causes irreversible tissue remodelling and organ dysfunction.

[0071] Thus, the present invention provides methods of treating fibrosis and fibrosis-related diseases using GSPION-peptide conjugates as described herein. As used herein, "fibrosis" refers to the thickening and/or scarring of connective tissue anywhere in the body. As used herein, a "fibrosis-related disease or condition" may be any disease or condition

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characterised by the presence of fibrosis, any disease or condition caused by fibrosis, or any disease or condition having fibrosis as a symptom thereof.

[0072] Peptides suitable for the treatment of fibrosis and fibrosis-related diseases and conditions may include relaxin family peptide receptor (RXFP) agonists. Thus, in an embodiment, the disease or condition to be treated or diagnosed is fibrosis or a fibrosis-related disease or condition and the therapeutic peptide is a relaxin family peptide receptor (RXFP) agonist, or an analogue thereof. RXFP agonists are known to treat fibrosis or a fibrosis-related diseases or conditions, for example, by intravenous infusion or intranasal delivery. The RXFP agonist may, for example, be an RXFP1, RXFP2, RXFP3 or RXFP4 agonist. In an embodiment, the RXFP agonist is a relaxin peptide or an analogue thereof. The relaxin peptide may be a relaxin 1 (RLX1) peptide, a relaxin 2 (RLX2) peptide or relaxin 3 (RLX3) peptide, preferably a RLX2 or RLX3 peptide.

[0073] Serelaxin (recombinant human gene-2 relaxin; RLX2) is the major stored and circulating form of relaxin, which binds to RXFP1 and was first identified for its ability to promote collagen-remodelling to enable separation of the pelvic ligaments of pregnant mammals and has now been well-studied for its anti-fibrotic properties in several organs within the body. In particular, serelaxin acts at multiple levels to inhibit fibrosis progression, via its anti-apoptotic, anti-inflammatory and anti-hypertrophic actions, and ability to inhibit the pro-fibrotic impact of various factors such as transforming growth factor (TGF)- β 1, connective tissue growth factor, angiotensin II and endothelin-1 on fibroblast to myofibroblast transition and myofibroblast-mediated ECM production. Furthermore, serelaxin is able to promote the balance between collagen-degrading matrix metalloproteinases (MMPs) and their tissue inhibitors of MMPs (TIMPs) that induces the MMP-induced resolution of established fibrosis, whilst also having vasodilatory and angiogenic actions.

[0074] Thus, in an embodiment, the RLX2 peptide is recombinant H2 relaxin (serelaxin).

[0075] In another embodiment, the RXFP receptor agonist is a relaxin peptide analogue, such as B7-33 or CGEN25009.

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[0076] Other suitable RXFP agonists will be apparent to those skilled in the art. Further, a skilled person will be able to readily identify the ability of a peptide or a peptide analogue to activate RXFP using routine assays known in the art, for example, ligand binding/competition assays or second messenger (cAMP, cGMP, ERK1/2) activity assays in cells (over-)expressing RXFPs, Surface Plasmon Resonance (SPR) and single-molecule FRET assays.

[0077] Further, the RXFP1 receptor can form a heterodimer with the angiotensin type 2 receptor (AT2R), which allows AT2R agonists to indirectly activate RXFP1. Thus, in another embodiment, the disease or condition to be treated or diagnosed is fibrosis or a fibrosis-related disease or condition and the therapeutic peptide is an AT2R agonist. Suitable AT2R agonists will be apparent to those skilled in the art and may include angiotensin II, angiotensin III, angiotensin 1-7, CGP42112, beta-Pro⁷-angiotensin III, and beta-Pro⁷-Trp⁸-angiotensin III, or analogues thereof. A skilled person will be readily able to identify the ability of a peptide or a peptide analogue to activate AT2R using routine assays known in the art, for example, ligand binding/competition assays or second messenger (cGMP, ERK1/2) activity assays in cells (over-)expressing the AT2R, ELISA, Mass spectrometry, SPR and FRET assays.

[0078] In an embodiment, the AT2R agonist is beta-Pro⁷-Trp⁸-angiotensin III.

[0079] Glycine-coated SPION-peptide conjugates comprising an RXFP agonist, an AT2R agonist, or an analogue thereof, or a combination thereof, may be used to treat or diagnose any form of fibrosis or fibrosis-related disease or condition. Non-limiting examples of fibrosis or fibrosis-related diseases or conditions include cardiomyopathy, interstitial lung fibrosis, liver fibrosis, non-alcoholic steatohepatitis (NASH), cirrhosis, pre-cirrhosis, diffuse parenchymal lung disease, cystic fibrosis, pulmonary fibrosis, progressive massive fibrosis, idiopathic pulmonary fibrosis, injection fibrosis, renal fibrosis, chronic kidney disease, diabetic kidney disease, focal segmental glomerulosclerosis, membranous nephropathy, IgA nephropathy, myelofibrosis, heart failure, metabolic heart failure, cardiac fibrosis, cataract fibrosis, cataract, ocular scarring, pancreatic fibrosis, skin fibrosis, intestinal fibrosis, intestinal strictures, endomyocardial fibrosis, atrial fibrosis, mediastinal fibrosis, Crohn's disease, retroperitoneal fibrosis, keloid, nephrogenic systemic fibrosis, scleroderma, systemic sclerosis, arthrofibrosis, Peyronie's syndrome, Dupuytren's contracture, diabetic

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neuropathy, adhesive capsulitis, alcoholic liver disease, hepatosteatorosis, viral hepatitis, biliary disease, primary hemochromatosis, drug-related cirrhosis, cryptogenic cirrhosis, Wilson's disease, alpha 1-antitrypsin deficiency, interstitial lung disease (ILD), human fibrotic lung disease, macular degeneration, retinal retinopathy, vitreal retinopathy, myocardial fibrosis, Grave's ophthalmopathy, drug induced ergotism, cardiovascular disease, atherosclerosis/restenosis, hypertrophic scars, primary or idiopathic myelofibrosis, inflammatory bowel disease and collagenous colitis.

[0080] In a particular embodiment, the fibrosis-related disease or condition is cardiomyopathy.

[0081] In a particular embodiment, the fibrosis is interstitial lung fibrosis.

[0082] The SPION-peptide conjugates disclosed herein may be administered to a subject in need of treatment (or diagnosis) for a disease or condition that is treatable (or diagnosable) with the peptide, or they may be administered in a prophylactic sense. In particular, it is clear that the methods of the invention may be used prophylactically as well as for the alleviation of symptoms of a disease or condition, such as fibrosis or a fibrosis-related disease or condition. References herein to "treatment" or the like may therefore include such prophylactic treatment, as well as therapeutic treatment of acute conditions or symptoms. Accordingly, in one or more embodiments, the present invention provides SPION-peptide conjugates for use in the therapeutic treatment (or diagnosis) of a disease or condition that is treatable (or diagnosable) with the peptide. In other embodiments, the present invention provides SPION-peptide conjugates for use in the prophylactic treatment of diseases or conditions that are treatable with the peptide.

[0083] The terms "treat", "treating" or "treatment" with regard to a disease or condition refers to alleviating or abrogating the cause and/or the effects of the disease or condition. As used herein, the terms "treat", "treatment" and "treating" refer to the reduction or amelioration of the progression, severity and/or duration of the disease or condition, or the amelioration of one or more symptoms (e.g., one or more discernible symptoms) of the disease or condition (i.e., "managing" without "curing" the condition), resulting from the administration of one or more therapies (e.g., one or more therapeutic agents such as a SPION-peptide conjugate as disclosed herein). In specific embodiments, the terms "treat",

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“treatment” and “treating” refer to the amelioration of at least one measurable physical parameter of a disease or condition. In other embodiments the terms “treat”, “treatment” and “treating” refer to the inhibition of the progression of a disease or condition, either physically by, e.g., stabilization of a discernible symptom or physiologically by, e.g., stabilization of a physical parameter, or both.

[0084] The terms “preventing” and “prophylaxis” as used herein refer to administering a medicament in order to avert or forestall the appearance of one or more symptoms of a disease or condition. The person of ordinary skill in the medical art recognises that the term “prevent” is not an absolute term. In the medical art, it is understood to refer to the prophylactic administration of a drug to substantially diminish the likelihood or seriousness of a disease or condition, or symptom of the disease or condition and this is the sense intended in the present disclosure. As used in a standard text in the field, the Physician’s Desk Reference, the terms “prevent”, “preventing” and “prevention” with regard to a disease or condition refer to averting the cause, effects, symptoms or progression of a disease or condition prior to the disease or condition fully manifesting itself.

[0085] The subject may be any animal in need to treatment and encompasses human and non-human subjects, including, but not limited to, mammals, birds and fish, and suitably encompasses domestic, farm, zoo and wild animals, such as, for example, cows, pigs, horses, goats, sheep or other hoofed animals, dogs, cats, chickens, ducks, non-human primates, guinea pigs, rabbits, ferrets, rats, hamsters and mice. Preferably, the subject is a mammal, more preferably a human.

[0086] The SPION-peptide conjugates disclosed herein are to be administered to the subject in need thereof in a treatment effective amount. In some embodiments, a treatment effective amount is a therapeutically effective amount or a prophylactically effective amount. The term “therapeutically effective amount” as used herein means an amount of SPION-peptide conjugate sufficient to treat or alleviate the symptoms associated with a disease or condition. The therapeutically effective amount of the compound to be administered will be governed by such considerations, and is either, an incremental maximum tolerated dose, or the minimum amount, necessary to ameliorate, cure, or treat the disease or condition or one or more of its symptoms. The term “prophylactically effective

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amount” refers to an amount effective in preventing or substantially lessening the chances of acquiring a disease or condition or in reducing the severity of the disease or condition before it is acquired or reducing the severity of one or more of its symptoms before the symptoms develop. Roughly, prophylactic measures are divided between primary prophylaxis (to prevent the development of a disease or symptom) and secondary prophylaxis (whereby the disease or symptom has already developed and the patient is protected against worsening of this process). Prophylaxis may include post-exposure prophylaxis (e.g., administering an effective amount of a SPION-peptide conjugate as disclosed herein to a subject known to be susceptible to a disease or condition that is treatable with the peptide). As used herein, the term “effective amount” relates to an amount of SPION-peptide conjugate which, when administered according to a desired dosing regimen, provides the desired therapeutic or diagnostic activity. For example, an effective amount of a SPION-peptide conjugate may be an amount sufficient to inhibit, slow, interrupt, halt, prevent or arrest a disease or condition that is treatable with the peptide. Suitable effective amounts may depend on the age, gender, weight and general health of the patient and can be determined by the attending physician or diagnostician. Suitable dosages may lie within the range of about 0.1 ng per kg of body weight to 100 g per kg of body weight per dosage. The dosage may be in the range of 1 µg to 10 g per kg of body weight per dosage, such as is in the range of 1 mg to 1000 mg per kg of body weight per dosage. In one embodiment, the dosage may be in the range of 1 mg to 500 mg per kg of body weight per dosage. In another embodiment, the dosage may be in the range of 1 mg to 250 mg per kg of body weight per dosage. In yet another embodiment, the dosage may be in the range of 1 mg to 200 mg per kg of body weight per dosage, such as up to 50 mg per kg body weight per dosage.

[0087] Where specific dosages or concentrations of SPION-peptide conjugate are referred to herein, it is to be understood that the specific dosage or concentration refers to the concentration of or equivalent to the neutral form of the peptide conjugated to the SPION. Accordingly, where a pharmaceutically acceptable salt of a peptide is used in the SPION-peptide conjugate, a person skilled in the art would readily understand that the concentrations or dosages in respect of the salt form of the peptide, refers to the equivalent concentration or dosage of the neutral form of the peptide.

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[0088] The terms “administer”, “administering” or “administration” in reference to a compound (i.e., SPION-peptide conjugate), composition or formulation disclosed herein means introducing the active agent (i.e., the SPION-peptide conjugate) into the system of the subject in need of treatment. When the active agent is provided in combination with one or more other active agents, “administration” and its variants are each understood to include concurrent and/or sequential introduction of the SPION-peptide conjugate and the other active agents.

[0089] In certain embodiments, the compositions disclosed herein may be provided at dosage levels sufficient to deliver from about 0.001 mg/kg to about 100 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, and from about 1 mg/kg to about 25 mg/kg, of subject body weight per day, one or more times a day, to obtain the desired therapeutic effect. In certain embodiments, an effective amount of a peptide for oral administration to a 70 kg adult human may comprise about 0.0001 mg to about 4000 mg, about 0.0001 mg to about 3000 mg, about 0.0001 mg to about 200 mg, about 0.001 mg to about 1500 mg, about 0.01 mg to about 1000 mg, about 0.1 mg to about 1000 mg, about 1 mg to about 1000 mg, about 1 mg to about 100 mg, about 10 mg to about 1000 mg, or about 100 mg to about 1000 mg, of an extract or compound per unit dosage form. In some embodiments, a single dose may be sufficient to treat or prevent a disease or condition that is treatable with the peptide. A single dose may be delivered in one or more aliquots (e.g., one or more tablets, capsules or gelcaps) to achieve the desired dose. In other embodiments, multiple doses may be required to treat or prevent the disease or condition. Dosing may occur at intervals of minutes, hours, days, weeks, months or years or continuously over any one of these periods. The administered amount may be an amount sufficient to treat or alleviate the symptoms associated with the relevant disease or condition.

[0090] The amount of peptide administered per dose or the total volume of composition administered may depend on such factors as the nature and severity of the symptoms, the age, weight, and general health of the patient, as well as the mode of administration. It is to be recognised that relative amounts of excipients, solvents, diluents, binders, disintegrants, lubricants, glidants and/or any additional ingredients in a pharmaceutical composition as

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disclosed herein may also depend upon the identity, size, and/or condition of the subject treated, as well as the mode of administration. For example, in some embodiments, the dosage of peptide required to achieve a therapeutically equivalent effect may be greater for solid oral dosage forms compared to liquid oral dosage forms. The terms “therapeutic equivalence” or “therapeutically equivalent” as used herein refer to different compositions comprising the same active agent that produce the same clinical effect and safety profile and/or are pharmaceutical equivalents to one another.

[0091] The SPION-peptide conjugates disclosed herein may be administered in a single dose or a series of doses. Suitable dosage amounts and dosing regimens can be determined by the attending physician and may depend on the particular condition being treated, the severity of the condition as well as the general age, health and weight of the subject. It will be appreciated that dose ranges as described herein provide guidance for the administration of provided pharmaceutical compositions to an adult. The amount to be administered can be determined by a medical practitioner or person skilled in the art.

[0092] The formulations disclosed herein may be administered to a subject in need thereof by any suitable oral delivery method, including but not limited to those described elsewhere herein. Suitable methods for oral administration would be well-known to a person skilled in the art.

[0093] In general, the SPION-peptide conjugate may be administered in a manner compatible with the route of administration and physical characteristics of the recipient (including health status) and in such a way that it elicits the desired effect(s). A SPION-peptide conjugate or a pharmaceutical composition, as described herein, may be administered to a recipient in isolation or in combination with other additional therapeutic agent(s). In embodiments in which a pharmaceutical composition comprising the SPION-peptide conjugate is formulated for administration with additional therapeutic agent(s), the administration may be simultaneous or sequential (i.e., administration of the SPION-peptide conjugate is followed by administration of the additional agent(s) or vice versa). Thus, where two or more entities are administered to a subject “in conjunction”, they may be administered in a single composition at the same time, or in separate compositions at the same time, or in separate compositions separated in time.

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[0094] In a non-limiting example, the SPION-peptide conjugates disclosed herein may be administered in conjunction with an active agent that is known to treat the same disease or condition as the peptide. For example, in embodiments in which the peptide is a relaxin peptide receptor (RXFP) agonist, an angiotensin type 2 receptor (AT2R) agonist, or an analogue thereof, and the disease or condition to be treated is fibrosis or a fibrosis-related disease or condition, the SPION-peptide conjugate may be administered in conjunction with a further anti-fibrotic agent. Suitable anti-fibrotic agents would be known to persons skilled in the art, illustrative examples of which include nintedanib and pirfenidone.

[0095] In some embodiments, a SPION-peptide conjugate as disclosed herein may be administered to a subject in need thereof, together with one or more other medications for a discrete period of time, to address specific symptoms of a disease or condition. In still other embodiments, the person in need thereof may be treated with a SPION-peptide conjugate and one or more additional medications (administered sequentially or in combination) for the duration of the treatment period. Such combination therapy may be particularly useful, for example, where an additive or synergistic therapeutic effect is desired.

[0096] The SPION-peptide conjugates disclosed herein may be used in combination therapy with one or more additional therapeutic agents. For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents may be administered separately or in conjunction. In addition, the administration of one element may be prior to, concurrent to, or subsequent to the administration of the other agent. The phrase “combination therapy” as used herein, is to be understood to refer to administration of an effective amount, using a first amount of, for example, a SPION-peptide conjugate as described herein, and a second amount of an additional suitable therapeutic agent.

[0097] When co-administered with another agent, an “effective amount” of the second agent will depend on the type of drug used. Suitable dosages are known for approved agents and can be adjusted by a person skilled in the art according to the condition of the subject, the type of condition(s) being treated and the amount of a compound, extract or composition being used. In cases where no amount is expressly noted, an effective amount should be assumed. For example, compounds described herein can be administered to a subject in a dosage range from between about 0.01 to about 10,000 mg/kg body weight/day, about

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0.01 to about 5000 mg/kg body weight/day, about 0.01 to about 3000 mg/kg body weight/day, about 0.01 to about 1000 mg/kg body weight/day, about 0.01 to about 500 mg/kg body weight/day, about 0.01 to about 300 mg/kg body weight/day, about 0.01 to about 100 mg/kg body weight/day.

[0098] In certain embodiments, the SPION-peptide conjugate and the additional therapeutic agent are each administered in an effective amount (i.e., each in an amount that would be therapeutically effective if administered alone). In other embodiments, the SPION-peptide conjugate and the additional therapeutic agent are each administered in an amount that alone does not provide a therapeutic effect (a sub-therapeutic dose). In yet other embodiments, the SPION-peptide conjugate can be administered in an effective amount, while the additional therapeutic agent is administered in a sub-therapeutic dose. In still other embodiments, the SPION-peptide conjugate can be administered in a sub-therapeutic dose, while the additional therapeutic agent is administered in an effective amount.

[0099] As used herein, the terms “in combination” or “co-administration” can be used interchangeably to refer to the use of more than one therapy (e.g., one or more prophylactic and/or therapeutic agents). The use of the terms does not restrict the order in which therapies (e.g., prophylactic and/or therapeutic agents) are administered to a person in need thereof.

[00100] Co-administration encompasses administration of the SPION-peptide conjugate and one or more additional therapeutic agents in an essentially simultaneous manner, such as in a single pharmaceutical composition, for example, a tablet, capsule or gelcap having a fixed ratio of first and second amounts, or as discrete dosage forms. In addition, such co-administration also encompasses use of each compound in a sequential manner in either order. When co-administration involves the separate administration of a first amount of a SPION-peptide conjugate and a second amount of an additional therapeutic agent, they are administered sufficiently close in time to have the desired therapeutic effect. For example, the period of time between each administration which can result in the desired therapeutic effect, can range from minutes to hours and can be determined taking into account the properties of each compound such as potency, solubility, bioavailability, plasma half-life, and kinetic profile.

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[00101] In one or more embodiments where the SPION-peptide conjugate is administered with an additional therapeutic agent, the additional therapeutic agent may be any therapeutic agent that provides a desired treatment outcome. In particular, the additional therapeutic agent may be selected from known therapeutic agents for the treatment or prevention of the disease or condition that is treatable using the relevant peptide (e.g., a RXFP agonist or AT2R agonist and fibrosis or a fibrosis-related disease or condition), including one or more symptoms thereof.

[00102] The SPION-peptide conjugates disclosed herein may, for example, be administered in combination with other therapeutic agents suitable for use in the treatment of fibrosis or fibrosis-related diseases or conditions, such as angiotensin-converting enzyme (ACE) inhibitors (e.g., benazepril, captopril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, trandolapril), angiotensin II receptor blockers (e.g., irbesartan, valsartan, losartan, telmisartan, olmesartan, candesartan), beta blockers (e.g., acebutolol, atenolol, bisoprolol, metoprolol, nadolol, nebivolol, propranolol), diuretics (e.g., chlorothiazide, chlorthalidone, hydrochlorothiazide, indapamide, metolazone, bumetanide, ethacrynic acid, furosemide, torsemide, amiloride, eplerenone, spironolactone, triamterene), corticosteroids (e.g., cortisone, hydrocortisone, prednisone), bronchodilators (e.g., salbutamol, salmeterol, formoterol, vilanterol, ipratropium, tiotropium, aclidinium, glycopyrronium, theophylline), mucolytics (e.g., acetylcysteine, carbocysteine, erdosteine, guaifenesin), antihistamines (e.g., brompheniramine, chlorpheniramine, clemastine, cyproheptadine, dexchlorpheniramine dimenhydrinate, diphenhydramine, doxylamine, hydroxyzine, phenindamine, azelastine, loratadine, cetirizine, desloratadine, fexofenadine, cimetidine, famotidine, nizatidine, ranitidine), angiotensin-receptor neprilysin inhibitors (e.g., sacubitril, valsartan), If channel blockers (e.g., ivabradine), SGLT-2 inhibitors (e.g., bexagliflozin, canagliflozin, dapagliflozin, empagliflozin, ertugliflozin), aldosterone antagonists (e.g., eplerenone, spironolactone), vitamins (e.g., vitamin E), oxidative stress inhibitors (e.g., thymoquinone), hepatic stellate cell (HSC) inhibitors, digoxin, nintedanib, pirfenidone, fedratinib, pacritinib, ruxolitinib, adalimumab, usekinumab, and the like. Other examples of suitable therapeutic agents will be apparent to those skilled in the art.

[00103] Where a SPION-peptide conjugate is administered in combination with an additional therapeutic agent, the second agent may be administered in any “effective

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amount” that provides the desired therapeutic activity, as described above. Suitable dosage amounts and dosing regimens of the additional therapeutic agent can be determined by the attending physician and may depend on the particular condition being treated, the severity of the condition as well as the general age, health and weight of the subject. It will be appreciated that, unless otherwise specified, dose ranges as described herein provide guidance for the administration of provided pharmaceutical compositions to an adult. The amount to be administered to can be determined by a medical practitioner or person skilled in the art.

[00104] The SPION-peptide conjugates and formulations thereof as disclosed herein may be contained in a kit. The kit may include, for example, a therapeutically (or diagnostically) effective amount of the SPION-peptide conjugate, optionally together with an additional agent, each packaged or formulated individually, or packaged or formulated in combination. Thus, the SPION-peptide conjugate may be present in first container (e.g., a bottle or blisterpack), and the kit can optionally include one or more agents in a second container. The container or containers may be placed within a package, and the package can optionally include administration or dosage instructions. For example the package may comprise a label attached to or packaged with the container, the label describing the contents of the container and providing indications and/or instructions regarding use of the contents of the container to treat a disease or condition treatable with the peptide. The kits may optionally comprise instructions describing a method of using the pharmaceutical compositions in one or more of the methods described herein (e.g., for preventing or treating fibrosis or fibrosis-related disease or condition using a glycine-coated SPION conjugated to a RXFP agonist or an AT2R agonist). The kit may optionally comprise a second pharmaceutical composition comprising one or more additional agents described herein for co-therapy use, and/or a pharmaceutically acceptable. The pharmaceutical composition comprising the SPION-peptide conjugate and the second pharmaceutical composition contained in the kit may be optionally combined in the same pharmaceutical composition.

[00105] Those skilled in the art will be aware that the disclosure provided herein is subject to variations and modifications other than those specifically described. It is to be understood that the disclosure provided herein includes all such variations and modifications. The present disclosure also includes all such steps, features, methods, compositions and

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compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

[00106] Certain embodiments of the invention will now be described with reference to the following examples which are intended for the purpose of illustration only and are not intended to limit the scope of the generality hereinbefore described.

EXAMPLES

Materials

[00107] Isoprenaline hydrochloride (ISO; I5627) and methacholine (A2251) were obtained from Merck/Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS; tlr1-eklps) was purchased from InvivoGen (San Diego, CA, USA). Recombinant H2 relaxin (serelaxin; RLX) was provided by Corthera Inc. (San Mateo, CA, US; a subsidiary of Novartis International AG; Switzerland).

Methods

Example 1. Synthesis and pharmacokinetics of SPION-conjugated RLX (SPION-RLX) and FITC-labelled SPION-RLX (SPION-RLX^{FITC})

[00108] Glycine-coated SPIONs were synthesized utilizing a modified alkaline co-precipitation method as described previously (Barick and Hassan, 2012; Chakraborty et al., 2019; Chakraborty et al., 2021). SPIONs were then conjugated to the N-terminus of RLX (at a concentration of 0.05mg/ml) using carbodiimide chemistry as described previously (Chakraborty et al., 2021). The pharmacokinetics of SPION-RLX had been evaluated previously (Chakraborty et al., 2021), where it was found that SPIONs could conjugate RLX up to 60ng/ml. Both 25ng (the dose used in the present study) and 60ng of SPION-RLX equivalently attenuated the expression levels of transforming growth factor (TGF)- β 1-stimulated collagen I from human lung myofibroblasts, after 72 hours in culture. Moreover, the maximum tolerant dose of SPIONs has been previously verified, where the cell viability of human A549 cells remained at 100% following treatment with up to 400 μ g/ml of SPIONs (Chakraborty et al., 2019). However, that study only utilized a SPION load of 200 μ g/ml to synthesize the 25ng/day of SPION-RLX used for animal studies. For the studies involving

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the LPS model, fluorescein isothiocyanate (FITC) was conjugated to the lysine residues of RLX using a previously described method (Masliah et al., 2011).

Example 2. Experimental design

[00109] 11-12-week-old, male C57BL/6J mice, weighing 26-30g were obtained from the Monash Animal Research Platform (MARF, Monash University, Clayton, Australia) and used to establish and treat the model of cardiomyopathy outlined below. Male mice were used as they are more susceptible to develop cardiomyopathy-induced heart failure (HF). On the other hand, 6-8-week-old female Balb/c mice (provided by MARF) were used to establish and treat the model of acute lung injury, as female Balb/c mice are more susceptible to developing airway inflammation compared to their male counterparts. In each case, mice were housed under standard conditions in the mouse facility of the Department of Pharmacology, with ad-libitum access to a standard chow diet (Barastock Stockfeeds, Pakenham, Australia) and water, on a 12-hour light/12-hour dark cycle. Mice were allowed to acclimatize for 6-7 days prior to being subjected to any experimental procedures. All experiments were approved by Monash University's Animal Ethics Committee (under MARF/2020/26910 or MARF/2021/ 29157) in line with the Australian Code of Practice for the Care and Use of Laboratory Animals for Scientific Purposes. Animals were randomized via a blinded analysis into groups of equal sizes: n=8 for the short-term (14-day) cardiomyopathy model; n=7 for the longer-term (42-day) cardiomyopathy model; and n=6 for the acute lung injury model. Power calculations were carried out to ensure that with a 20% standard deviation, the studies conducted would be 80% powered, to detect a 20-35% effect, with n=6-8 animals per group, respectively.

Example 3. Establishment and treatment of the isoprenaline-induced model of cardiomyopathy

[00110] The non-invasive 14-day model of isoprenaline (ISO)-induced cardiomyopathy (Brooks and Conrad, 2009; Wang et al., 2021), was utilised in this study. Sub-groups of mice were subjected to once-daily subcutaneous administrations of ISO (25mg/kg body weight (BW)) for five consecutive days. Mice were then left untreated for a further 9 days (until day 14) for fibrotic healing to occur (injury control/ISO group). ISO is a synthetic catecholamine and β -adrenoceptor agonist that stimulates a positive chronotropic effect in

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the heart. Hence, when repeatedly administered over 5 days, the heart undergoes an aberrant wound healing-induced fibrosis owing to its limited capacity to heal. This eventually resulted in LV remodelling and dysfunction by day 14. A separate group of mice, which were injected once-daily with saline for five consecutive days and left untreated until day 14, were included as a non-injury control group (**Figure 1**).

[00111] In addition to the two control groups established, four separate sub-groups of ISO-injured mice received various treatments from day 7 to 14 post-injury. One sub-group was subjected to the subcutaneous implantation of osmotic minipumps containing RLX (0.5mg/kg/day; which continuously infused RLX into the circulation of mice, at a dose that was bioactive in mice; ISO+Pump-RLX group). This dose of RLX had been reported to produce circulating H2 relaxin levels of ~15-25ng/ml by 5-14 days post-administration (Samuel et al., 2003), which was equivalent to the supraphysiological RLX levels found in pregnant women producing twin or triplet foetuses at the same time (Bell et al., 1987). Two separate sub-groups of ISO-injured mice were administered with 25ng/day of SPION-conjugated RLX (SPION-RLX; which represented the higher end of circulating RLX levels produced by Pump-RLX). RLX was conjugated to SPIONs as described previously (Chakraborty et al., 2021), and this dose of SPION-RLX equivalently reduced measures of airway inflammation, remodelling, fibrosis and hyperresponsiveness (AHR) to that of Pump-RLX when intranasally (i.n)-administered to mice with chronic allergic airways disease. SPION-RLX was administered either via i.p injections every 72 hours (on days 7, 10 and 13; ISO+SPION-RLX (i.p) group) or via daily drinking water, from days 7-14 post-injury, in 15ml Falcon tubes with stoppers (ISO+SPION-RLX (p.o) group). The concentration of SPION-RLX provided to the latter group of mice was calculated based on mice drinking ~4-5ml of water each day. To ensure all mice drank a similar volume of water containing SPION-RLX, these mice were individually housed. Another separate subgroup of ISO-injured mice was also administered empty nanoparticles (ISO+Empty-SPIONs) that were not conjugated to RLX, either i.p (n=4) or p.o (n=4) (**Figure 1**). All mice underwent systolic blood pressure (SBP) measurements on days 0, 6 and 13, and heart rate (HR) measurements on day 13. Furthermore, all mice with the exception of ISO+Empty-SPION group, also underwent transthoracic echocardiography on day 14. Mice were then humanely killed by cardiac puncture on day 14, for blood (plasma) and tissue collection.

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[00112] The longer-term effects of orally-administered SPION-RLX and SPION-B7-33 (a B-chain analogue of RLX, which contains the receptor binding domain of RLX for the RXFP1 receptor) were also compared to the effects of a current standard of care medication, the angiotensin converting enzyme (ACE) inhibitor, perindopril, in a 42 day ISO-induced model. In this study, separate sub-groups of mice were subjected to once-daily subcutaneous administrations of ISO (25mg/kg BW) for five consecutive days. Mice were then left untreated for a further 37 days (until day 42) for fibrotic healing to occur (injury control/ISO group). A separate group of mice, which were injected once-daily with saline for five consecutive days and left untreated until day 42, were included as a non-injury control group.

[00113] In addition to the two control groups established, six separate sub-groups of ISO-injured mice received various treatments via oral gavage (p.o), every 3 days from day 14 to 42 post-injury. Two sub-groups were administered with 25ng/day of SPION-conjugated RLX (SPION-RLX) or SPION-conjugated B7-33 (SPION-B7-33), respectively. An additional sub-group was administered 60ng/day of perindopril. The three remaining sub-groups were administered 25ng/day of unconjugated RLX alone, 25ng/day of unconjugated B7-33 alone or Empty SPIONs alone, respectively (which were established as negative control groups) (**Figure 2**).

Example 4. Establishment and treatment of the lipopolysaccharide-induced model of acute lung injury

[00114] To assess the therapeutic effects of SPION-RLX and its interaction with its cognate receptor, RXFP1, a lipopolysaccharide (LPS)-induced model of acute lung injury (ALI) was established in 6-8-week-old female BALB/c mice. On day 0, mice were briefly anaesthetised via isoflurane (Aerrane; 2-3% in oxygen; Baxter Healthcare, Toongabbie, New South Wales, Australia) then sensitised with i.n-administered LPS (50µl of a 5µg/ml solution; 250ng/mouse). LPS was administered intranasally to directly induce lung damage, influx of pro-inflammatory cells and emphysema. One sub-group of LPS-sensitized mice (injury control/LPS group) were left untreated until day 7 (a time-point at which they developed significant lung inflammation and related AHR). An uninjured control group of mice were administered 50µl of saline (SAL) intranasally on day 0 instead of LPS, and were left untreated until day 7. Two separate sub-groups of LPS-instilled mice were randomly allocated to two treatment groups consisting of SPION-RLX (25ng/day; a dose previously

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used to demonstrate therapeutic effects, and which produced ~20-22ng/ml of circulating RLX when i.n-administered; LPS+SPION-RLX group) or Empty-SPIONs (LPS+Empty-SPION group). SPION-RLX or Empty-SPIONs were i.n-administered every 48 hours on days 2, 4 and 6 post-LPS-induced lung injury (**Figure 3**). On day 7 post-LPS or saline-administration, all mice underwent a lung function test (by invasive plethysmography), prior to being killed for tissue collection.

Example 4a. Establishment and treatment of bleomycin-induced model of lung fibrosis

[00115] In a 28-day bleomycin (BLM)-induced model of lung fibrosis, sub-groups of 11-12-week-old male C57BL/6 mice were intranasally instilled via their nares (nose) with saline (50uL per day; healthy control group) or bleomycin (BLM; 0.15mg in 50uL per day; injury control group) on day 0 and day 7, then mice left for a further 21 days for lung fibrosis to develop. On day-21 post-injury (14-days after the 2nd instillation of BLM), sub-groups of BLM-instilled mice (n=8 per group) were treated with: a 7-day minipump-infused AT2R agonist (beta-Pro⁷-Trp⁸-angiotensin III) at a dose of 0.1mg/kg/day (17.5ug of the AT2R agonist in total over days 21-28); oral gavage administration of the AT2R agonist alone (17.5ug per administration on day 21, 24 and 27; negative control); oral gavage administration of the glycine-coated SPION-AT2R agonist (17.5ug per administration on day 21, 24 and 27); oral gavage administration of Empty-SPIONs alone (on day 21, 24 and 27) (**Figure 14**). All mice were then killed at day 28 for tissue collection.

Example 5. Subcutaneous implantation of osmotic minipumps

[00116] Mice were initially anaesthetized with isoflurane (2-3% in oxygen) using an anaesthetic induction chamber. Once anaesthetized, mice were maintained in a supine position and an 8-10mm incision was then made between the scapulae, so that a skin pocket could be created with blunt scissors. The minipump (model 1007D, Alzet®, Cupertino, CA, USA; with had an infusion rate of 0.5µl/hour for 7 days) was then implanted into the skin pocket with the open end of the pump facing the tail of the mouse. The incision site was then closed with Michel clips and mice were monitored until they regained consciousness.

Example 6. Oral gavage administration of treatments

[00117] At the appropriate time, mice were restrained using the scruff hold and a gavage needle/tubing (22-gauge curved needlex38mm tubing) was inserted into the mouth of each mouse. The needle/tubing was gently advanced into the mouth at the back of the throat to locate the oesophageal entrance at the back of the pharynx; and twilled at the oesophageal entrance to encourage the mouse to swallow. The needle/tubing was advanced gently into the oesophagus and into the stomach to the required distance, a small volume of fluid (from a total of 150-200 μ l of each treatment) injected into the mouse to ensure that the needle was intragastric, and the remaining volume injected (if the initial fluid injected did not appear at the mouth or nose). Mice were observed for 5 minutes after the procedure had been completed, then returned to their cages.

Example 7. Measurement of haemodynamic parameters using tail cuff plethysmography

[00118] To investigate the effects of ISO-induced cardiomyopathy and the subsequent treatments evaluated on hemodynamic parameters, HR and SBP were measured using tail cuff plethysmography (MC4000 Blood Pressure System; Hatteras Instruments Inc., USA). HR was measured on day 13, while SBP was measured prior to the initiation of injury (on day 0), prior to commencing any treatment (on day 6), and prior to killing mice for tissue collection (on day 13). In line with previous studies, 15-20 SBP measurements of both HR and SBP were obtained to achieve a pooled mean for each animal (Wang et al., 2021; Samuel et al., 2014).

Example 8. Evaluation of cardiac function using transthoracic echocardiography

[00119] To elucidate the effects of ISO and the subsequent treatments evaluated on cardiac (LV) stiffness and function, transthoracic echocardiography was performed using the Vevo 2100 Imaging system (Visual Sonics Inc., Toronto, Canada). Echocardiography was carried out at the Monash Biomedical Imaging facility (Clayton, Victoria, Australia), on all groups of mice (with the exception of the ISO+Empty-SPION group) at day 14 post-saline or ISO administration. Echocardiographic measurements were obtained from grey-scale two-dimensional (2D) B-mode images acquired in the parasternal long-axis view and M-mode images at the midpapillary level in the parasternal short-axis view. The animal was then

tilted backwards in the Trendelenburg position to obtain the four-chamber view through the apex of the heart to perform pulsed-wave Doppler imaging.

Example 9. Evaluation of airway function using invasive plethysmography

[00120] On day 7 post-saline or LPS administration to mice, all animals were subjected to invasive plethysmography to analyse AHR. Prior to the procedure, mice were anaesthetised with ketamine (100mg/kg BW) and xylazine (20mg/kg BW) prior to tracheostomy and cannulation. Mice were then placed in the FinePointe whole body plethysmography chamber (Buxco Electronics, Troy, NY, USA) and exposed to doubling concentrations (from 3.125 to 50 mg/ml) of the bronchoconstrictor, methacholine (MCh) via nebulisation. AHR was measured for three minutes (per dose of MCh), calculated based on difference from baseline (nebulised PBS) following each MCh dose, and expressed as airway resistance.

Example 10. Tissue, plasma and bronchoalveolar lavage fluid (BALF) isolation

[00121] On day 14 post-saline or -ISO administration, all mice were initially weighed and subsequently killed via cardiac puncture following an overdose of isoflurane (5% in oxygen). Approximately ~500-700µL of blood was withdrawn and placed into a heparinised tube (Minicollect Greiner Bio-One, Kremsmünster, Austria). Heparinised tubes were centrifuged at 4°C for 10 minutes at 12,000rpm for the isolation and collection of plasma, which was stored at -80°C until required for the quantification of circulating RLX levels. The heart was then isolated, blot-dried and weighed (heart weight (HW)), and the atria and right ventricle were trimmed off to isolate the LV. The LV was then separately weighed (LV weight (LVW)) and transversely sectioned into the apex, mid-zone, and base. The apex was repeatedly washed in Dulbecco's phosphate-buffered saline (dPBS) to remove any blood cells in preparation for flow cytometry analysis. The base was snap frozen in liquid nitrogen and stored at -80°C until required for protein extraction analyses. The mid-zone was fixed in 10% neutral buffered formalin (NBF) to be processed for histological and immunohistochemical (IHC) analyses. For the groups treated with SPIONs, the liver was also isolated and fixed in 10% NBF in a cassette. Both the mid-zone and liver sections were then sent to Monash Histology Platforms for tissue processing.

[00122] On day 7 post-saline or LPS administration (and following AHR measurements), bronchoalveolar lavage fluid (BALF) was collected from each anaesthetised mouse with

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three repeated washes (400µl each). The BALF was spun in a centrifuge for 4 minutes at 4°C at 1500rpm to isolate cells. Each supernatant was removed and was subject to a protein assay. BALF cells were treated with ACK (ammonium and potassium citrate) buffer to lyse red blood cells, then resuspended in FACS buffer and stored on ice for future cell counting. Mice were then culled for lung tissue removal, which was weighed, and separated into four separate lobes. The largest lobe was fixed in 10% NBF overnight and subsequently sent to Monash Histology Platform to be processed, embedded in paraffin wax, and sectioned for analysis of tissue histopathology. The second largest lobe was prepared for FACS analysis, whilst the remaining two lobes were snap-frozen in liquid nitrogen and stored at -80°C.

Example 11. ELISA analysis of circulating RLX levels (ISO model)

[00123] To correlate the circulating RLX levels with its therapeutic effects, RLX levels in the plasma of mice treated with Pump-RLX, SPION-RLX (i.p or p.o) or Empty-SPIONs were quantified using the H2 RLX Quantikine ELISA kit (DRL200, R&D Systems, Minneapolis, USA). The ELISA was performed according to the manufacturer's instructions, with all standards and samples assayed in duplicates, as described in Chakraborty et al., 2021.

Example 12. Histological staining and analysis

[00124] Serial 5µm LV sections (from mice subjected to saline, ISO or BLM) were stained with H&E (to measure LV inflammation) or 0.1% picosirius red (Polysciences, Inc, Warrington, PA, USA; to measure interstitial LV fibrosis, LV cardiomyocyte hypertrophy or interstitial lung fibrosis). An additional serial LV section along with 5µm liver sections from ISO-injured mice and lung section from BLM-injured mice were also stained with Perl's Prussian blue staining (which can detect the iron core of SPIONs; to identify the distribution of SPIONs in these organs) and counterstained with neutral red. A 5µm lung section (from mice subjected to saline or LPS) were stained with H&E (to measure lung inflammation). All staining was performed by the Monash Histology Platform (Clayton, Victoria, Australia), and all stained slides were then digitally scanned using the Aperio Scanscope AT Turbo scanner (Leica Biosystems, Nußloch, Germany), whereby the high-resolution images were stored on a local server associated with the instrument.

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[00125] The morphometric analysis of various end-points was then performed in a blinded fashion using the Aperio ImageScope v.12.4.3 software (Leica Biosystems). Given the low levels of LV inflammation detected in ISO-injured mice, the infiltration of inflammatory cells into the LV myocardium was semi-quantified from 10 random and non-overlapping fields of view (FOV) at x200 magnification. These cell counts were then pooled to obtain the mean number of inflammatory cell counts for each animal. Given the higher levels of lung inflammation observed in LPS-injured mice, 10 random and non-overlapping FOV per mouse were randomly selected and scored for inflammation using a score of 0-4 (where 0 = no inflammation; 1 = 25% or occasional leukocytes with scar exudates; 2 = 50% or several leukocytes with clear exudates; 3 = 75% leukocytes with high degree of septal thickening full of exudate; 4 = 100% or severe inflammation with distended alveoli filled with leukocytes; Royce et al., 2014). The picrosirius red-stained interstitial LV collagen deposition was determined from 10 random and non-overlapping FOV (at x200 magnification) per section and expressed as a fraction (%) of the total area stained. To quantify cardiomyocyte hypertrophy, picrosirius red-stained LV sections were morphometrically analysed at x400 magnification. Within each section analysed, the area of 10 random cells from 10 random non-overlapping FOV was traced and measured, to obtain a mean cardiomyocyte area per section from 100 non-overlapping cardiomyocytes. The blue-stained SPIONs from the Perl's Prussian blue staining were just visualised for their distribution within the LV and liver.

Example 13. IHC staining for markers of LV inflammation, fibrosis and angiogenesis

[00126] Immunohistochemistry was performed to quantify the expression of pro-inflammatory and profibrotic markers within the injured LV myocardium (of saline- and ISO-injected mice). Separate serial mid-zone sections were stained with either a polyclonal IgG antibody to tumour necrosis factor (TNF)- α (ab6671; 1:250 dilution), interleukin (IL)-1 β (ab205924; 1:500 dilution) or TGF- β 1 (a pro-fibrotic cytokine; ab92486; 1:250 dilution; all from Abcam Antibodies, Cambridge, MA, USA); or a monoclonal IgG2A clone 1A4 antibody to α -SMA (a marker of myofibroblast differentiation and smooth muscle-associated blood vessel density; M0851; 1:1000 dilution; Agilent Technologies (Dako), Mulgrave, Victoria, Australia). The following day, the respective sections were stained with either Dako Envision⁺ System kits containing either a HRP-labelled anti-rabbit secondary

antibody (K4003; for the detection of TNF- α , IL-1 β or TGF- β 1) or HRP-labelled anti-mouse secondary antibody (K4000; for the detection α -SMA). Antibody binding was visualised by 3,30-diaminobenzidine (DAB; Dako), before slides were counterstained with haematoxylin and mounted in DePex (VWR International, Radnor, PA, USA).

[00127] IHC-stained slides were also scanned using the Aperio Scanscope AT Turbo scanner and analysed in a blinded fashion using the Aperio ImageScope v.12.4.3 software. For TGF- β 1 staining, the strong positive DAB (brown)-staining from 10 random and non-overlapping FOV (at x200 magnification) per section was detected and expressed as a fraction (%) of the total area stained. For TNF- α , IL-1 β and α -SMA, the number of positive DAB-stained cells per FOV were counted at magnifications of x400, x100 or x200, respectively, and expressed as the number of positively-stained cells per field. Additionally, the number of α -SMA-stained blood vessel density was counted from 10 random and non-overlapping FOV (at x100 magnification) per section, to provide a measure of vascular rarefaction in each of the groups evaluated.

Example 14. Flow cytometry analysis of immune cell influx

[00128] To elucidate the effects of ISO administration and the subsequent treatments evaluated on immune cell populations within the LV, populations of regulatory T cells (Tregs), M2-like macrophages and dendritic cells (DCs) were quantified by flow cytometry, as described previously (Chakraborty et al., 2021). To elucidate the effects of LPS administration and the subsequent treatments evaluated in the latter model within the airways/lung, populations of M1-like macrophages and DCs were quantified by flow cytometry instead. The cells from the LV apex or second largest lung lobe were isolated and resuspended in a FACS buffer (dPBS+ 5% FCS+ 0.5mM EDTA), from which FACS sorting was carried out on 1×10^5 cells/ml. Initially, samples were incubated with a rat anti-mouse CD16/32 Fc block (#553141; 1:100 dilution; BD Horizon, NJ, USA) to prevent non-specific Fc binding. Cells were then stained with fluorescently-labelled primary antibodies (as detailed in **Table 1**). Another set of cells from a saline-treated mouse were left unstained to act as the unstained control. For gating specific cell subsets, three fluorescent minus one controls (FMOs) were prepared. Subsets of cells were either stained with 1) all primary antibodies except FoxP3 (FMO1); 2) all antibodies except CD45 (FMO2); or 3) all antibodies except for CD206 (FMO3). All cells were washed in FACS buffer before

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proceeding to live/dead staining. Subsequently, all cellular subsets except the unstained control and FMOs were subjected to secondary staining with Zombie aqua dye (#423101; 1:1000 dilution; BioLegend, San Diego, CA, USA) for live/dead screening. For intracellular FoxP3 staining, all cells were permeabilised with a permeabilization buffer (dPBS+1% Triton-X) and incubated in the dark for 15 minutes. The cells were washed and resuspended in dPBS containing 0.1% Triton-X followed by the addition of FoxP3 primary antibody (prepared in permeabilization buffer). Cells were subsequently incubated in the dark for 20 minutes and were washed in FACS buffer, before re-fixing occurred in 1% paraformaldehyde. Single cell controls were prepared utilizing compensation beads (AbCTM Total antibody compensation bead kit; #A10513, Thermo Fisher Scientific, Waltham, MA, USA). Corresponding to each channel in the flow-cytometer, the primary antibodies mentioned in **Table 1** were conjugated along with negative beads. Cells were then acquired on a BD Fortessa X-20 Cytometer (FlowCore Platform, Monash University, Clayton, Victoria, Australia). While the unstained control was utilized to calibrate the voltages for forward and side scatter (FSC and SSC, respectively), the single cell controls were used to setup compensation and voltages for each channel. Data were analysed using Flow Jo TM v10.8 (BD Biosciences, San Jose, CA, USA). Initially, gating was performed as FSC-A and SSC-A, followed by FSC-H and FSC-A for doublet discrimination/exclusion. The single cells obtained were used for live/dead screening and live cells (zombieneg), and were used for further analysis. Using the FMOs and unstained cells, macrophages were gated as CD45⁺ cells followed by gating of F4/80⁺CD206⁻ cells as M1-like macrophages versus F4/80⁺CD206⁺ cells as M2-like macrophages. Tregs were gated and classified as CD4⁺CD25⁺Foxp3⁺ cells, whilst DCs were gated as CD11c⁺ cells.

Table 1: Details of the primary antibodies used for flow cytometry analysis.

Antibody	Cat. No	Source	Dilution used
CD45-PE-Cy5 (anti-mouse)	553082	BD Biosciences, San Jose, CA, USA	1:200
CD4-BUV496 (anti-mouse)	741050	BD Biosciences, San Jose, CA, USA	1:200
CD25-BV785 (anti-mouse)	564368	BD Biosciences, San Jose, CA, USA	1:100
Foxp3-V450-BV421 (anti-mouse)	561293	BD Biosciences, San Jose, CA, USA	1:100

Antibody	Cat. No	Source	Dilution used
F4/80-APC-Fire750 (anti mouse)	123151	BioLegend, San Diego, CA, USA	1:100
CD206-AF647 (anti mouse)	141711	BioLegend, San Diego, CA, USA	1:100
CD11c-BUV395 (anti mouse)	564080	BD Horizon, Franklin Lakes, NJ, USA	1:100

Example 15. Statistical analysis

[00129] All data were expressed as the mean $\pm$ standard error of the mean (SEM). Data analysis was performed using GraphPad Prism v9.1.2, from group sizes of $n=6-8$ per group, where n denotes the number of independent samples obtained from each group. Changes in SBP and AHR were analysed using a two-way ANOVA (to assess the effects of treatment vs time for SBP; or treatment vs MCh concentration tested for AHR), whilst all other endpoints were analysed using a one-way ANOVA. Post-hoc comparisons between groups were only carried out if the p -value of the overall ANOVA was statistically significant (i.e. $p<0.05$) and there was no significant variance in homogeneity. A Bonferroni post-hoc test was applied to the two-way ANOVA, whilst a Tukey's post-hoc test was applied to the one-way ANOVA to allow for multiple comparisons to be made between the appropriate groups. For the (MMP, TIMP and MMP:TIMP ratio) data that was normalised to the saline control group, all individual values from each group (including those from the saline control group) were normalised to the mean of the saline group, which was expressed as 1 in each case. In this case, these data were analysed using a nonparametric (Kruskal–Wallis) test and Dunn's post-hoc test. All data were included unless any values were >2 SDs from the mean. No approaches were used to reduce unwanted sources of variation by data normalization or to generate normal data. Differences were considered statistically significant at $p<0.05$, and the threshold value was not varied during the study.

Results and discussion

The systemic or oral administration of SPION-RLX attenuated established cardiomyopathy-induced LV inflammation by abrogating immune cell infiltration and pro-inflammatory cytokine expression, and promoting dendritic cell recruitment

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[00130] Given that LV inflammation is the initial response to myocardial injury, H&E-stained sections were morphometrically analysed to assess total inflammatory cell infiltration into the LV, whilst immunohistochemical analysis of LV proinflammatory cytokines and flow cytometry analysis of immune cell subsets within the LV were also evaluated as measures of the immune system's response to cardiac injury. Compared to measurements obtained from saline-treated control mice (average of 304 ± 7 cells per field), there was a ~27% increase in inflammatory cell infiltration within the LV of isoprenaline (ISO)-injured mice with cardiomyopathy, at day 14 post-injury (387 ± 11 cells per field; $p < 0.001$ vs saline group; **Figure 4a, 4b**). In line with these findings, ISO-injured mice had a ~1-fold ($\sim 20 \pm 2$ cells per field; **Figure 4c**) and ~5.7-fold ($\sim 10 \pm 1$ cells per field; **Figure 4d**) increase in the number of TNF- α - and IL-1 β -expressing cells within the LV at day 14 post-injury, compared to respective measurements obtained from saline-treated mice ($\sim 10 \pm 2$ TNF- α -expressing cells per field; $\sim 1.5 \pm 1$ IL-1 β -expressing cells per field; both $p < 0.001$ vs saline group). This ISO-induced increase in LV inflammation (characterized by inflammatory cell infiltration as well as TNF- α - and IL-1 β -expressing cells) was equivalently abrogated by Pump-RLX (322 ± 14 cells/ $\sim 11 \pm 1$ TNF- α -expressing cells/ $\sim 3 \pm 1$ IL-1 β -expressing cells per field), i.p.-injected SPION-RLX (346 ± 8 cells/ $\sim 10 \pm 1$ TNF- α -expressing cells/ $\sim 4 \pm 1$ IL-1 β -expressing cells per field) or p.o.-administered SPION-RLX (347 ± 7 cells/ $\sim 12 \pm 1$ TNF- α -expressing cells/ $\sim 4 \pm 1$ IL-1 β -expressing cells per field) after 7 days of treatment (all $p < 0.05$ vs ISO-treatment alone; all $p < 0.05$ vs ISO+Empty-SPION treatment). However, these ISO-induced inflammatory measures were unaffected by Empty-SPION treatment over the same time-period (408 ± 7 cells/ $\sim 17 \pm 2$ TNF- α -expressing cells/ $\sim 8 \pm 1$ IL-1 β -expressing cells per field; all $p < 0.01$ vs respective measurements from saline-treated mice; all no different to respective measurements from ISO-injured mice; **Figure 4a-d**).

[00131] Flow cytometric analysis of LV tissues from ISO-injured mice revealed that these mice had a significant (~40-50%) reduction in the proportion of infiltrating CD11c⁺ dendritic cells (DCs; $5.2 \pm 0.7\%$; **Figure 4e, 4f**) and similar reduction in CD11C⁺ CD206⁺ monocyte-derived DCs ($3.4 \pm 0.6\%$; **Figure 4e, 4g**) within the myocardium in comparison to respective measurements obtained from saline-treated controls ($8.1 \pm 0.6\%$ and $6.7 \pm 1.5\%$, respectively; $p < 0.05$ vs saline group for CD11c⁺ DCs; **Figure 4f**). Conversely, these ISO-injured mice

had a significant increase in F4/80⁺ CD206⁺ M2-like macrophage (6.1±0.7%; **Figure 5a**) and CD4⁺ CD25⁺ FoxP3⁺ regulatory T cell (Treg; 6.7±0.8%; **Figure 5b**) infiltration by day 14 post-injury compared to respective measurements obtained from saline-treated controls (3.5±0.6% and 1.2±0.1%, respectively; both p<0.05 vs saline group). Strikingly, RLX treatment, either through continuous Pump administration or sporadic SPION-RLX administration, significantly promoted the infiltration of DCs into the injured LV (by 2-3.5-fold for CD11c⁺ and CD11c⁺ CD206⁺ DCs; all p<0.05 vs the ISO group) after 7 days of treatment; an effect that was not induced by empty-SPIONs alone over the same time period (**Figure 4f, 4g**). Furthermore, all forms of RLX treatment evaluated significantly ameliorated the ISO-induced increase in CD4⁺ CD25⁺ FoxP3⁺ Treg infiltration within the LV after 7 days (by ~60-75%; all p<0.05 vs ISO group; all no different to saline group; **Figure 5b**); which again was not induced by empty-SPIONs alone. On the other hand, only Pump-RLX abrogated the ISO-induced increase in LV F4/80⁺ CD206⁺ M2-like macrophage infiltration within the LV (3.4±0.7%; **Figure 5a**).

[00132] Circulating (H2) RLX levels were quantified from the plasma of mice that were treated with continuous Pump-RLX (0.5mg/kg/day) vs systemic administration of SPION-RLX (25ng/day, via i.p injections or drinking water (p.o)) from days 7 to 14 post-injury. Plasma RLX levels in mice treated with pump-RLX (17.0±0.7ng/ml) were significantly different to that measured from mice treated with sporadically administered SPION-RLX via i.p injections (16.0±1.2ng/ml) or p.o-administration (14.5±0.8ng/ml) (**Figure 6**). These findings confirmed that similar levels of circulating RLX could be obtained via the continuous infusion of RLX through a minipump or, alternatively, through i.p or p.o-administered SPION-RLX.

The intranasal administration of SPION-RLX attenuated acute LPS-induced lung inflammation following its uptake by dendritic cell and M1 macrophage infiltration

[00133] The LPS-induced murine model of acute lung injury (ALI) was confirmed by LPS-instilled mice presenting with significantly increased airway/lung inflammatory cell infiltration (by ~1.5-fold; with a interstitial lung inflammation score of 3.40±0.10 vs 1.37±0.38 in saline-instilled controls; **Figure 7a, 7b**); and emphysema (**Figure 7c**) with a mean linear intercept (MLI) index of 17.5±1.3 vs 23.8±1.0 for saline-instilled mice compared to respective measurements from saline-treated controls (all p<0.01 vs. saline

group), by 7-days post-administration. The LPS-induced increase in inflammation score was markedly ameliorated by i.n-SPION-RLX treatment (by ~83%; 1.72 ± 0.24 ; $p < 0.001$ vs LPS alone group), but not empty-SPIONs alone (3.19 ± 0.30 ; no different to LPS alone group; **Figure 7b**). In line with these findings, the LPS-induced reduction of MLI index was completely abrogated by i.n-SPION-RLX treatment (23.6 ± 1.8 ; $p < 0.001$ vs LPS alone group), but was unaffected by empty-SPION treatment (17.9 ± 1.7 ; **Figure 7c**).

[00134] Upon analysis of proinflammatory cytokines secreted into the airways/lungs of LPS-instilled mice, these mice had significantly increased levels of TNF- α (by ~9-fold; **Figure 7d**), IL-1 β (by ~6.3-fold; **Figure 7e**) and IL-6 (by ~12.8-fold) in their lungs compared to respective measurements obtained from saline-instilled mice (all $p < 0.01$ vs saline-instilled group). In line with these findings, LPS-instilled mice had significantly increased thymic stromal lymphopoietin protein (TSLP)-associated airway epithelial damage (by ~17.6-fold; $p < 0.001$ vs saline-instilled group) compared to that measured in saline-instilled control mice. i.n-SPION-RLX treatment significantly attenuated all three proinflammatory cytokine levels (by ~70-80%) by preventing inflammatory cell influx; and also restored the LPS-induced airway epithelial damage (by ~80%) (all $p < 0.05$ vs LPS alone; **Figure 7d-g**). These effects of SPION-RLX were not induced by i.n-administration of empty-SPIONs, however.

[00135] The proportions of infiltrating pro-inflammatory cells such as DCs and M1 macrophages were evaluated by FACS analysis, to determine the sub-types of inflammatory cells that were contributing to LPS-induced airway/lung inflammation. To identify the proportion of DCs, viable BALF cells were gated for CD11c⁺ cells. The DC population was ~9.5-fold higher ($60.9 \pm 1.3\%$; $p < 0.001$ vs. saline group) in LPS-instilled mice compared to respective measurements from saline-instilled controls ($5.8 \pm 0.4\%$) after 7 days (**Figure 7h, 5i**). This LPS-induced increase in DC population was completely abolished by SPION-RLX ($3.5 \pm 0.1\%$; $p < 0.001$ vs LPS group) but was unaffected by empty-SPION treatment ($33.4 \pm 3.8\%$; **Figure 7i**). The T cell activating costimulatory molecule CD80 was used as an additional marker of activated cells. CD80 expression was decreased on DCs by ~0.97-fold of the mean fluorescence intensity (MFI) compared to respective measurements from the saline-instilled group after 7-days ($p < 0.05$ vs saline group; **Figure 7j**). On the other hand, BD80 MFI was markedly elevated by SPION-RLX treatment (by ~7-fold of levels measured in saline-instilled mice), but was only restored to levels measured in saline-instilled mice by

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empty-SPION treatment (**Figure 7j**); suggesting that these DCs were predominantly taking up the i.n-administered SPION-RLX.

[00136] To identify the proportion of macrophages that had infiltrated the airways/lungs of LPS-instilled mice, BALF cells were gated for CD45⁺ monocytes. Of this population, pro-inflammatory M1-like macrophages were identified as F4/80⁺ CD206⁻ cells, whereas alternatively activated M2-like macrophages were identified as F4/80⁺ CD206⁺ cells. There was a greater proportion of infiltrating M1-like macrophages (95±2%) compared to M2-like macrophages (5±2%) following LPS-instillation in comparison to that measured from saline-instilled controls (**Figure 7k-m**). Pro-inflammatory M1-like macrophage infiltration was significantly increased (by ~8.4-fold; **Figure 7l**), while anti-inflammatory M2-like macrophage infiltration was significantly reduced (by ~0.4-fold; **Figure 7m**) in the airways/lungs of LPS-instilled mice compared to that measured in their saline-instilled counterparts (both $p < 0.001$ vs saline control group). The LPS-induced influx of M1 macrophages was completely attenuated by SPION-RLX, but only modestly attenuated by empty-SPION treatment (**Figure 7l**). On the other hand, the LPS-induced reduction in M2-like macrophage infiltration was fully restored by SPION-RLX, but not empty-SPION treatment (**Figure 7m**).

DCs and M1-like macrophages were found to express the relaxin receptor, RXFP1, and were involved in the uptake of SPION-RLX in LPS-instilled mice

[00137] The uptake of FITC-tagged SPION-RLX by immune cells in LPS-instilled mice was localised to the cytoplasm of DCs and M1-like macrophages, as observed by FACS analysis (**Figure 8a-c**) and histological staining (stained cells indicated by arrows in **Figure 8d**). FACS analysis indicated that the uptake of SPION-RLX by DCs was ~75% greater than that observed in saline-instilled controls (**Figure 8b**), whilst that by alveolar M1-like macrophages was ~46% greater than that observed in saline-instilled controls (**Figure 8c**). Expression of Relaxin Family Peptide Receptor 1 (RXFP1; the cognate receptor for RLX) was upregulated in DCs (**Figure 8b**) and M1-like macrophages (**Figure 8c**) following LPS-stimulation and treatment with SPION-RLX, compared to a concentration matched isotype control (FMO3-in blue), which gated for the negative population of RXFP1 absent cells and catered to any false-positive non-specific binding by the antibody isotype. This indicated SPION-RLX was able to target RXFP1 on DCs and M1-like macrophages to

mediate its effects in mice with acute lung injury. This uptake of SPION-RLX by immune cells within the damaged airway/lung of LPS-instilled mice allowed RLX to abrogate the LPS-induced increase in airway hyperresponsiveness/reactivity; an effect that could not be induced by empty-SPIONs alone (**Figure 8e**).

The systemic or oral administration of SPION-RLX attenuated established cardiomyopathy-induced LV fibrosis by attenuating pro-fibrotic TGF- β 1 activity, cardiomyocyte hypertrophy and vascular rarefaction, and promoting the balance between MMPs and TIMPs

[00138] Repeated ISO administration to mice induced a significant increase (by ~ 1.5 -fold) in interstitial LV collagen deposition (fibrosis) in these animals at 14 days post-injury ($4.0 \pm 0.4\%$), compared to respective measurements obtained from their saline-treated counterparts ($1.6 \pm 0.2\%$; $p < 0.01$ vs saline group; **Figure 9a, 9b**). This ISO-induced increase in LV fibrosis was accompanied by significantly increased interstitial LV myofibroblast accumulation (by ~ 2.2 -fold; ~ 30 cells/field vs ~ 9 cells/field in saline-injected mice; **Figure 9c**) and TGF- β 1 expression levels (by ~ 1 -fold; $1.2 \pm 0.1\%$ vs $0.6 \pm 0.1\%$ in saline-injected mice; **Figure 9d**) (both $p < 0.05$ vs saline group) at the time-point studied. The sporadic systemic (i.p) or oral administration of SPION-RLX to ISO-injured mice was able to abrogate the interstitial LV collagen fibrosis (to $\sim 2.0 \pm 0.3\%$; **Figure 9b**), myofibroblast accumulation (to 9-10 cells/field; **Figure 9c**) and TGF- β 1 expression levels (to $0.7 \pm 0.1\%$; **Figure 9d**) to an equivalent extent as Pump-RLX treatment, and to levels that were no different to that measured in saline-injected control mice. However, these therapeutic effects of SPION-RLX were not induced by empty-SPIONs alone.

[00139] Cardiac hypertrophy is an important feature of cardiac remodelling which positively correlates with LV fibrosis. As previous studies had shown that ISO-injured mice undergo changes in LV cardiomyocyte hypertrophy in the absence of any overt changes to heart weight or LV weight to body weight ratio (Wang et al., 2021), changes in LV cardiomyocyte size were assessed. Morphometric analysis of picrosirius red stained-LV sections revealed a significant increase in the LV cardiomyocyte cross-sectional area in ISO-injured mice (by $\sim 26\%$; $434 \pm 12 \mu\text{m}^2$) in comparison to measurements obtained from their saline counterparts ($345 \pm 12 \mu\text{m}^2$; $p < 0.01$ vs saline group; **Figure 9e, 9f**). This ISO-induced increase in cardiomyocyte cross-sectional area was abrogated following treatment with RLX

administered via a mini-pump ($345 \pm 17 \mu\text{m}^2$) or by SPION-RLX when administered either i.p ($363 \pm 14 \mu\text{m}^2$) or via drinking water ($354 \pm 13 \mu\text{m}^2$) (all by ~ 80 - 100% ; all $p < 0.05$ vs ISO alone). Notably, the cardiomyocyte cross-sectional area of all three RLX-treated groups was not significantly different to that measured in the saline-treated control group (all no different to saline controls). On the contrary, the ISO-induced increase in cardiomyocyte cross-sectional area was not affected by empty-SPION treatment ($422 \pm 19 \mu\text{m}^2$; $p < 0.01$ vs saline group; no different to the ISO-alone group; **Figure 9f**).

[00140] Cardiac hypertrophy is closely associated with vascular rarefaction within the myocardium. Cardiac hypertrophy can lead to the decreased perfusion of the cardiac muscle, which is one of the main sources of hypoxia-induced apoptosis in HF. Hence, given the increased cardiomyocyte size observed in ISO-injured mice, changes in blood vessel density were thus assessed in the various groups evaluated. Accordingly, there was a $\sim 36\%$ reduction in the myocardial blood vessel density in ISO-injured mice (7 ± 0.4 vessels per field) in comparison to their saline-treated counterparts (11 ± 0.6 ; $p < 0.01$ vs saline group; **Figure 9g, 9h**). However, all three forms of RLX treatment, either through pump-RLX (15 ± 0.3 vessels per field) or SPION-RLX administered i.p (15 ± 1 vessels per field) or p.o (15 ± 1 vessels per field) equivalently restored this ISO-induced loss of blood vessel density (all by ~ 1 -fold over the ISO alone group; all $p < 0.01$ vs ISO alone; **Figure 9h**). Notably, all RLX-treated groups restored blood vessel density to levels that were $\sim 30\%$ higher than that measured in saline-treated controls (all $p < 0.05$ vs saline group). These findings are in agreement with RLX's well-documented angiogenic effects (Sarwar et al., 2017; Unemori et al., 2000; Samuel et al., 2011). Conversely, the ISO-induced decrease in blood vessel density was unaffected by empty-SPION-treatment (6 ± 0.4 vessels per field; $p < 0.01$ vs saline group; no different to the ISO-alone group; **Figure 9h**).

[00141] In addition to evaluating measures of LV inflammation, hypertrophy and vascular rarefaction, which can all contribute to aberrant ECM production, factors that control the rate of ECM degradation were also measured in the ISO model established. ISO-injured mice underwent a significant loss of collagen-degrading matrix metalloproteinase (MMP)-13 (collagenase-3; the predominant collagenase in rodents; **Figure 10a, 10b**), MMP-9 (gelatinase B; **Figure 10a, 10c**) and MMP-2 (gelatinase A; **Figure 10a, 10d**) (all by ~ 30 - 40%) levels, but a significant increase in tissue inhibitor of metalloproteinase (TIMP)-1 (by

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~60%; **Figure 10a, 10e**) and TIMP-2 (by ~85-90%; **Figure 10a, 10f**) expression levels within the LV by 14 day post-injury. As a result of these findings, ISO-injured mice presented with a significant reduction in LV MMP-13/TIMP-1 (by ~60%; **Figure 10g**), MMP-9/TIMP-1 (by ~55%; **Figure 10h**) and MMP-2/TIMP-2 (by ~65%; **Figure 10i**) ratios at day 14 post-injury, in line with the increased interstitial LV fibrosis (**Figure 9a, 9b**) that was evident in these mice at the time-point studied. Pump-RLX treatment did not directly affect the ISO-induced loss of LV MMP-13 levels (**Figure 10b**), but restored the ISO-induced loss of LV MMP-9 (**Figure 10c**) and MMP-2 (**Figure 10d**) levels, significantly reduced the ISO-induced increase in LV TIMP-1 levels ($p<0.05$ vs ISO alone group; **Figure 10e**) and blunted the ISO-induced increase in LV TIMP-2 expression (**Figure 10f**) to levels that were no longer different to that measured in the LV of saline-injected control mice. This resulted in Pump-RLX treatment restoring the ISO-induced loss of LV MMP-13/TIMP-1, MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios (**Figure 10g-i**) to levels that were equivalent or no longer different to that measured in saline-injected control mice. On the other hand, whilst SPION-RLX (administered i.p or p.o) did not affect the ISO-induced loss of LV MMP-13 levels (**Figure 10b**), SPION-RLX administration was able to significantly promote LV MMP-9 (by ~1-1.80-fold over levels measured in the ISO alone group; **Figure 10e**) and LV MMP-2 (by ~1.1-1.2-fold over levels measured in the ISO alone group; **Figure 10f**) levels whilst being able to normalize the ISO-induced increase in TIMP-1 (**Figure 10e**) and TIMP-2 (**Figure 10f**) (all $p<0.05$ vs ISO alone group). This resulted in SPION-RLX administration being able to restore the ISO-induced loss of LV MMP-13/TIMP-1 ratio (**Figure 10g**) to levels measured in saline-injected control mice, but significantly increase the LV MMP-9/TIMP-1 (**Figure 10h**) and TIMP-2 (**Figure 10i**) (by ~3-5-fold over levels measured in the ISO alone group; all $p<0.01$ vs respective measurements from the ISO alone group; and ~0.6-1.3-fold over levels measured in saline-injected mice). These findings indicated that the SPION-RLX could promote the balance between collagen-degrading MMPs and their inhibitors (TIMPs) that would facilitate the MMP-induced degradation of established ECM, independently of the route of administration applied.

The systemic or oral administration of SPION-RLX attenuated the cardiomyopathy-induced LV dysfunction

[00142] To assess the ability of SPION-RLX to restore the ISO-induced LV dysfunction in mice, transthoracic echocardiography was performed on saline- and ISO-injected control mice and ISO-injured mice treated with Pump-RLX or SPION-RLX (administered i.p or p.o). Compared to measurements obtained from saline-treated control mice, ISO-injured mice underwent a significant reduction in ejection fraction ($47.3 \pm 1.5\%$ vs $58.1 \pm 3.5\%$ in saline-injected control mice) and a significant increase in end systolic volume ($26.8 \pm 1.3 \mu\text{L}$ vs $20.8 \pm 1.8 \mu\text{L}$) (both $p < 0.05$ vs saline-injected control group; **Table 2**). ISO-injured mice also underwent presented with a trend towards an increased end-diastolic volume ($53.6 \pm 3.5 \mu\text{L}$ vs $46.1 \pm 1.7 \mu\text{L}$, isovolumetric contraction time ($19.0 \pm 2.0 \text{ms}$ vs $15.9 \pm 0.9 \text{ms}$) and isovolumetric relaxation time ($22.1 \pm 1.3 \text{ms}$ vs $18.6 \pm 1.6 \text{ms}$) (**Table 2**), indicating that they had stiffened hearts. The ISO-induced loss of ejection fraction, and increase in end systolic and diastolic volume were equivalently restored by Pump-RLX or SPION-RLX treatment, indicating that SPION-RLX could also restore the functional deficits identified in ISO-injured mice, independently of the route of administration applied.

Table 2: The effects of ISO-induced cardiomyopathy and Pump-RLX versus SPION-RLX treatment on cardiac functional parameters

Parameter measured	SAL (n=7)	ISO (n=7)	ISO+ Pump-RLX (n=7)	ISO+SPION-RLX (i.p) (n=7)	ISO+ SPION-RLX (p.o) (n=7)
End systolic LV mass (mg)	45.6 $\pm$ 3.7	58.8 $\pm$ 3.9	43.4 $\pm$ 3.6	42.1 $\pm$ 2.7	42.3 $\pm$ 3.1
End diastolic LV mass (mg)	44.3 $\pm$ 3.1	53.2 $\pm$ 2.6	41.5 $\pm$ 3.0	43.3 $\pm$ 2.1	43.7 $\pm$ 2.7
LVPWT (systole) (mm)	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1
LVPWT (diastole) (mm)	0.8 $\pm$ 0.1	0.8 $\pm$ 0.1	0.8 $\pm$ 0.1	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1
Stroke volume (μL)	24.2 $\pm$ 2.4	27.9 $\pm$ 3.9	21.9 $\pm$ 1.8	24.2 $\pm$ 1.7	23.0 $\pm$ 1.1
Ejection fraction (%)	58.1 $\pm$ 3.5	47.3 $\pm$ 1.5*	56.2 $\pm$ 1.8 [#]	56.0 $\pm$ 2.2 [#]	56.9 $\pm$ 1.1 [#]
Fractional Shortening (%)	25.6 $\pm$ 2.2	22.3 $\pm$ 1.4	29.0 $\pm$ 2.0	29.0 $\pm$ 1.8	27.5 $\pm$ 0.7
End systolic volume (μL)	20.8 $\pm$ 1.8	26.8 $\pm$ 1.3*	18.2 $\pm$ 1.8 ^{##}	19.2 $\pm$ 1.6 [#]	17.9 $\pm$ 1.1 ^{##}
End diastolic volume (μL)	46.1 $\pm$ 1.7	53.6 $\pm$ 3.5	40.1 $\pm$ 3.3 [#]	41.9 $\pm$ 2.8 [#]	40.8 $\pm$ 1.3 [#]
E/A wave ratio	1.5 $\pm$ 0.1	1.5 $\pm$ 0.1	1.6 $\pm$ 0.1	1.5 $\pm$ 0.1	1.4 $\pm$ 0.1
IVCT (ms)	15.9 $\pm$ 0.9	19.0 $\pm$ 2.0	21.5 $\pm$ 2.2	19.2 $\pm$ 1.1	22.7 $\pm$ 1.8
IVRT (ms)	18.6 $\pm$ 1.6	22.1 $\pm$ 1.3	20.0 $\pm$ 2.4	21.2 $\pm$ 0.1	16.5 $\pm$ 1.4

Cardiac functional parameters measured on day 14 post-ISO-injury using transthoracic echocardiography are expressed as the mean $\pm$ standard error of the mean (SEM), from saline (SAL)-treated controls, ISO-injured mice alone and ISO-injured

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mice treated with either Pump-RLX (0.5mg/kg/day; from day 7-14 post-ISO injury), intraperitoneally (i.p)-administered SPION-RLX (25ng/day; on days 7, 10 and 13 post-ISO-injury) or daily drinking water (p.o)-administered SPION-RLX (25ng/day; from days 7-14 post-ISO-injury). ISO-injured mice presented with significant systolic dysfunction at day 14 post-injury, in that they underwent a significant reduction in ejection fraction (the amount of blood pumped out by the heart each time it beats) and corresponding increase in end-systolic and end-diastolic volume (which results from an increased afterload; the increased pressure the heart must work against to eject blood at systole and diastole). These ISO-induced measures of systolic dysfunction were equivalently abrogated by Pump-RLX and i.p- or p.o-administered SPION-RLX. The data presented were obtained from n=7 mice per group. *P<0.05 vs the saline (SAL) group; #P<0.05, ##P<0.01 vs the ISO group; as determined by a one-way ANOVA followed by Tukey's post-hoc test, which allowed for multiple comparisons between the groups shown. LVPWT (systole): LV posterior wall thickness at systole; LVPWT (diastole): LV posterior wall thickness at diastole; IVCT: isovolumetric contraction time, IVRT: isovolumetric relaxation time.

The conjugation of SPIONs to RLX (SPION-RLX) or B7-33 (SPION-B7-33) maintained the long-term therapeutic effects of these peptides when orally-administered, and provided broader cardioprotective effects compared to the ACE inhibitor, perindopril

[00143] Repeated ISO administration to mice induced a significant increase in interstitial LV fibrosis (by ~1.75-fold; **Figure 11**), LV cardiomyocyte size/hypertrophy (by ~30%; **Figure 12**) and LV vascular rarefaction (loss of blood vessel density; by ~30%; **Figure 13**) at 42 days post- injury, compared to respective measurements obtained from their saline-treated counterparts. The ISO-induced increase in interstitial LV fibrosis was fully abrogated by p.o-administered SPION-RLX or SPION B7-33 treatment, partially but significantly reduced by p.o-administered perindopril treatment (by ~40%), but unaffected by the p.o-administration of unconjugated RLX alone, unconjugated B7-33 alone or Empty SPIONs alone after 4-weeks of treatment (**Figure 11a, 11b**). As a result of this, p.o-administered SPION-B7-33 was able to reduce the ISO-induced increase in interstitial LV fibrosis to a significantly greater extent than p.o-administered perindopril after 4-weeks of treatment (**Figure 11b**). The ISO-induced increase in LV cardiomyocyte size/hypertrophy was equivalently abrogated by p.o-administered SPION-RLX, SPION-B7-33 or perindopril treatment, but was unaffected by the p.o-administration of unconjugated RLX alone, unconjugated B7-33 alone or Empty SPIONs alone after 4-weeks of treatment (**Figure 12a, 12b**). However, the ISO-induced LV vascular rarefaction was equivalently restored by p.o-administered SPION-RLX or SPION-B7-33 treatment, but was unaffected by p.o-administered perindopril, unconjugated RLX alone, unconjugated B7-33 alone or Empty SPIONs alone after 4-weeks of treatment (**Figure 13a, 13b**). These findings confirmed 1) that only the conjugation of RLX or B7-33 to SPIONs allowed for the cardioprotective

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activity of these peptides to be maintained over a 4-week period when p.o.-administered to mice with ISO-induced cardiomyopathy, whereas p.o.-administered (unconjugated) RLX alone, (unconjugated) B7-33 alone or Empty-SPIONs alone did not induce any cardioprotective effect on their own. 2) SPION-B7-33 provided improved anti-fibrotic efficacy compared to p.o.-administered perindopril (**Figure 11b**); whilst both p.o.-administered SPION-RLX and SPION-B7-33 provided improved angiogenic effects (on new blood vessel formation) compared to p.o.-administered perindopril (**Figure 13b**), suggesting that these SPION-conjugated peptides provided broader cardioprotection compared to perindopril.

The systemic or oral administration of a SPION-AT2R agonist attenuated BLM-induced interstitial lung fibrosis

[00144] BLM-injured mice underwent a 2.5-fold increase in picrosirius red-stained interstitial lung fibrosis at day 28 post-injury. This BLM-induced increase in interstitial lung fibrosis was significantly and equivalently reduced by the Pump-infused AT2R agonist or orally-administered glycine-coated SPION-AT2R agonist (by 70-75%), but not by the orally-administered AT2R agonist alone or Empty SPIONs alone, when these treatments were administered from days 21-28 post-injury (**Figure 15**).

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CLAIMS

1. A method of orally delivering a peptide to a subject, the method comprising conjugating the peptide to a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) to provide a SPION-peptide conjugate, and orally delivering the SPION-peptide conjugate to the subject.
2. The method of claim 1, wherein the peptide is a therapeutic peptide, a diagnostic peptide, or an analogue thereof.
3. The method of claim 1 or claim 2, wherein the peptide is a relaxin peptide receptor (RXFP) agonist.
4. The method of claim 3, wherein the RXFP agonist is a relaxin peptide or an analogue thereof.
5. The method of claim 4, wherein the relaxin peptide is a relaxin 2 (RLX2) peptide or a relaxin 3 (RLX3) peptide.
6. The method of claim 5, wherein the RLX2 peptide is recombinant H2 relaxin (serelaxin).
7. The method of claim 4, wherein the relaxin peptide analogue is B7-33 or CGEN25009.
8. The method of claim 1 or claim 2, wherein the peptide is an angiotensin type 2 receptor (AT2R) agonist.
9. The method of claim 8, wherein the AT2R agonist is selected from the group consisting of angiotensin II, angiotensin III, angiotensin 1-7, CGP42112, beta-Pro⁷-angiotensin III and beta-Pro⁷-Trp⁸-angiotensin III.
10. The method of claim 8 or claim 9, wherein the AT2R agonist is beta-Pro⁷-Trp⁸-angiotensin III.
11. The method of any one of claims 1 to 10, wherein the peptide has an atomic mass of about 170 kDa or less, preferably from about 3 kDa to about 70 kDa.

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12. The method of any one of claims 1 to 11, wherein the glycine is D-glycine.
13. The method of any one of claims 1 to 12, wherein the peptide is covalently coupled to the glycine via an amide bond.
14. The method of any one of claims 1 to 13, wherein the molar ratio of glycine-coated SPION to peptide is from about 4:1 to about 20:1.
15. The method of any one of claims 1 to 14, wherein the SPION-peptide conjugate has a hydrodynamic diameter from about 20 nm to about 100 nm.
16. The method of any one of claims 1 to 15, wherein conjugating the peptide to the glycine-coated SPION improves the oral bioavailability of the peptide.
17. Use of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a peptide for orally delivering the peptide to a subject.
18. A method of treating a disease or condition in a subject, the method comprising orally administering to the subject an effective amount of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a therapeutic peptide, wherein the disease or condition is treatable with the therapeutic peptide.
19. The method of claim 18, wherein the disease or condition is fibrosis or a fibrosis-related disease or condition and the therapeutic peptide is a relaxin peptide receptor (RXFP) agonist, an angiotensin type 2 receptor (AT2R) agonist, or an analogue thereof.
20. The method of claim 19, wherein the fibrosis or fibrosis-related disease or condition is selected from the group consisting of cardiomyopathy, interstitial lung fibrosis, liver fibrosis, non-alcoholic steatohepatitis (NASH), cirrhosis, pre-cirrhosis, diffuse parenchymal lung disease, cystic fibrosis, pulmonary fibrosis, progressive massive fibrosis, idiopathic pulmonary fibrosis, injection fibrosis, renal fibrosis, chronic kidney disease, diabetic kidney disease, focal segmental glomerulosclerosis, membranous nephropathy, IgA nephropathy, myelofibrosis, heart failure, metabolic heart failure, cardiac fibrosis, cataract fibrosis, cataract, ocular scarring, pancreatic fibrosis, skin fibrosis, intestinal fibrosis, intestinal strictures, endomyocardial fibrosis, atrial fibrosis, mediastinal fibrosis, Crohn's disease, retroperitoneal fibrosis, keloid, nephrogenic systemic fibrosis, scleroderma, systemic

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sclerosis, arthrofibrosis, Peyronie's syndrome, Dupuytren's contracture, diabetic neuropathy, adhesive capsulitis, alcoholic liver disease, hepatosteatorosis, viral hepatitis, biliary disease, primary hemochromatosis, drug-related cirrhosis, cryptogenic cirrhosis, Wilson's disease, alpha 1-antitrypsin deficiency, interstitial lung disease (ILD), human fibrotic lung disease, macular degeneration, retinal retinopathy, vitreal retinopathy, myocardial fibrosis, Grave's ophthalmopathy, drug induced ergotism, cardiovascular disease, atherosclerosis/restenosis, hypertrophic scars, primary or idiopathic myelofibrosis, inflammatory bowel disease and collagenous colitis.

21. The method of claim 19 or claim 20, wherein the fibrosis-related disease or condition is cardiomyopathy.

22. The method of claim 19 or claim 20, wherein the fibrosis is interstitial lung fibrosis.

23. A method of diagnosing a disease or condition in a subject, the method comprising orally administering to the subject an effective amount of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a diagnostic peptide, wherein the disease or condition is diagnosable with the diagnostic peptide.

24. Use of a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a therapeutic peptide, in the manufacture of a medicament for treating a disease or condition in a subject by oral administration, wherein the disease or condition is treatable with the therapeutic peptide.

25. An oral dosage form comprising a glycine-coated superparamagnetic iron oxide nanoparticle (SPION) conjugated to a peptide.

26. The oral dosage form of claim 25, which is a tablet, a capsule or a gelcap.

Figure 1

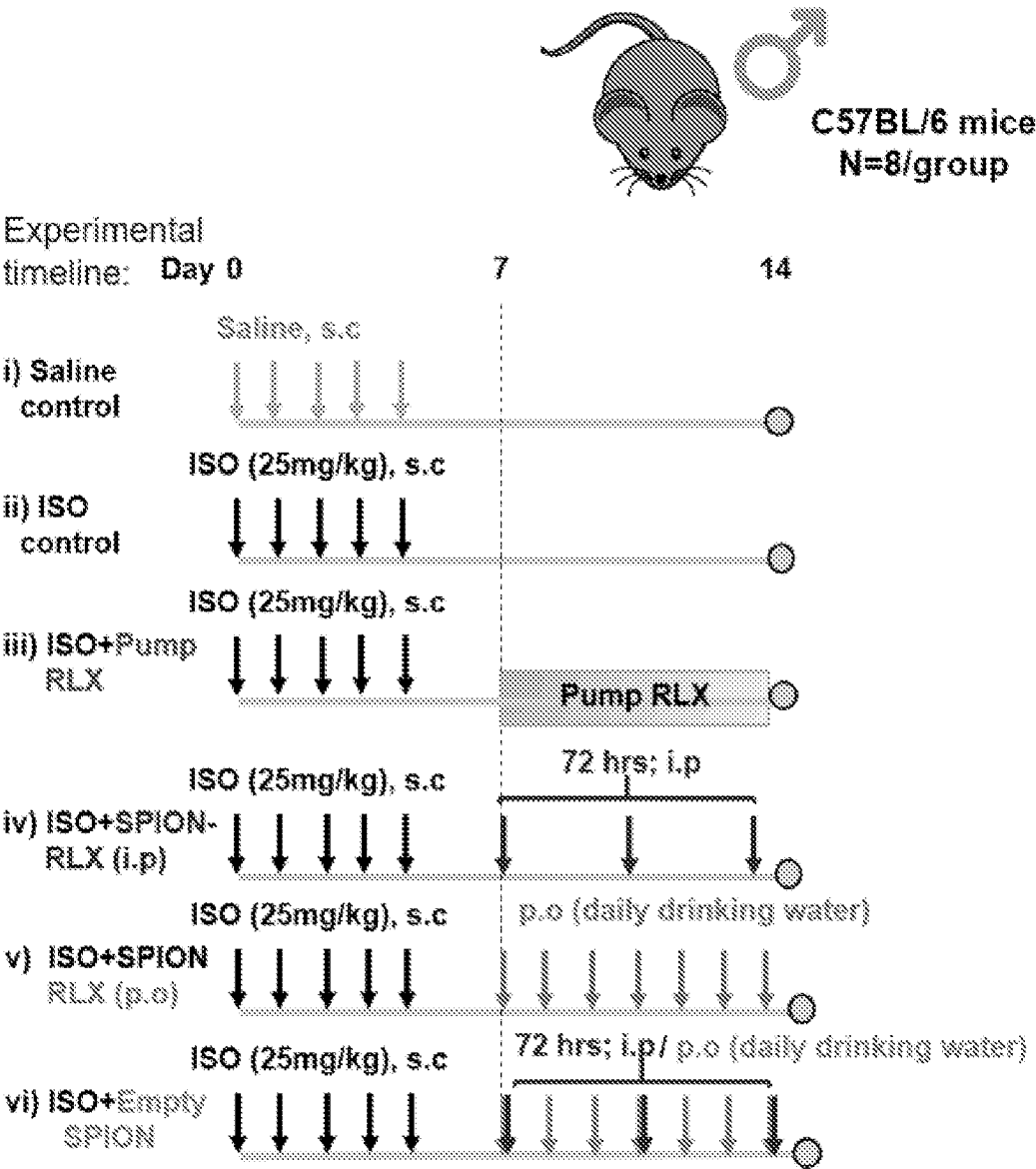
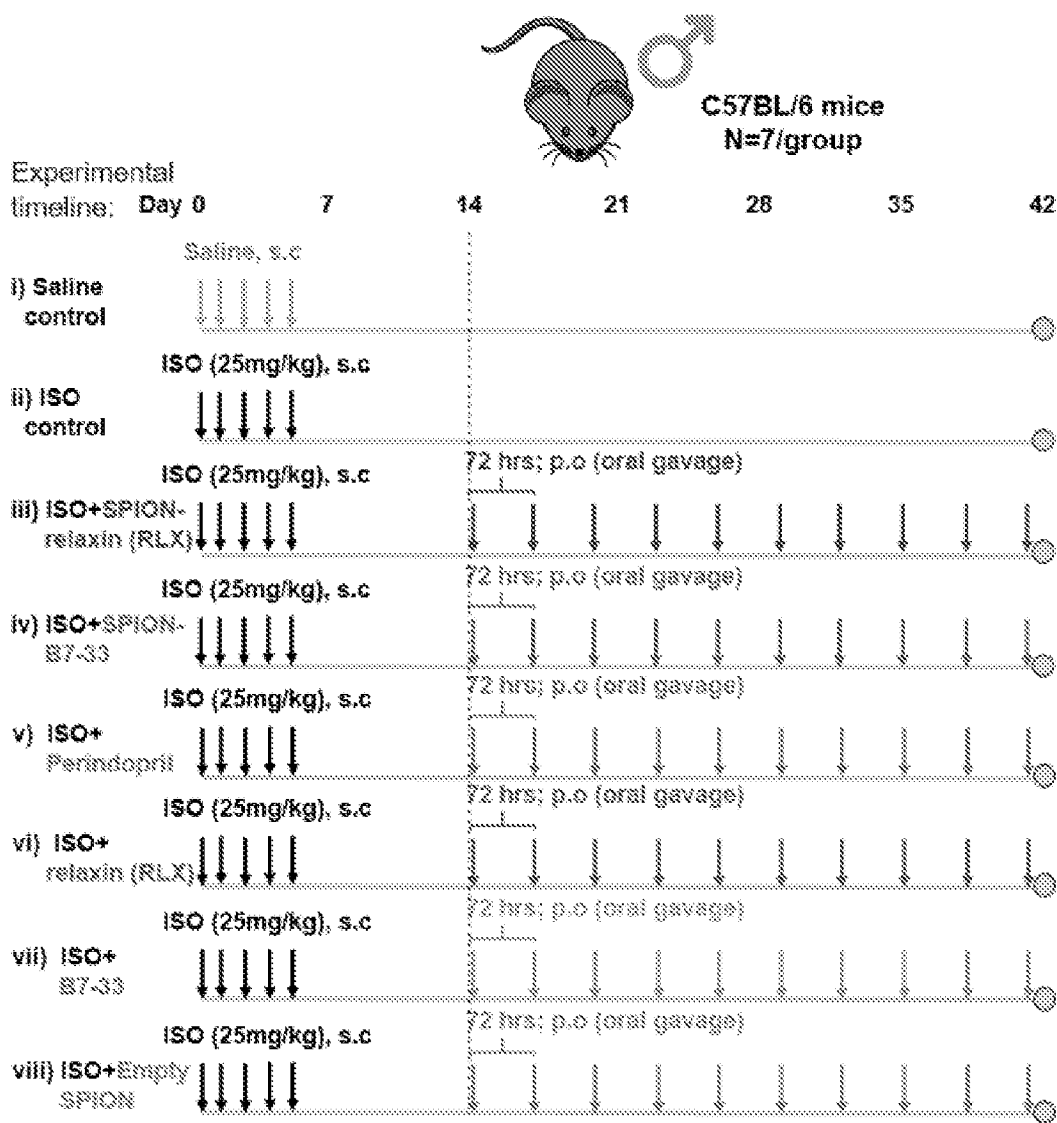


Figure 2



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Figure 3

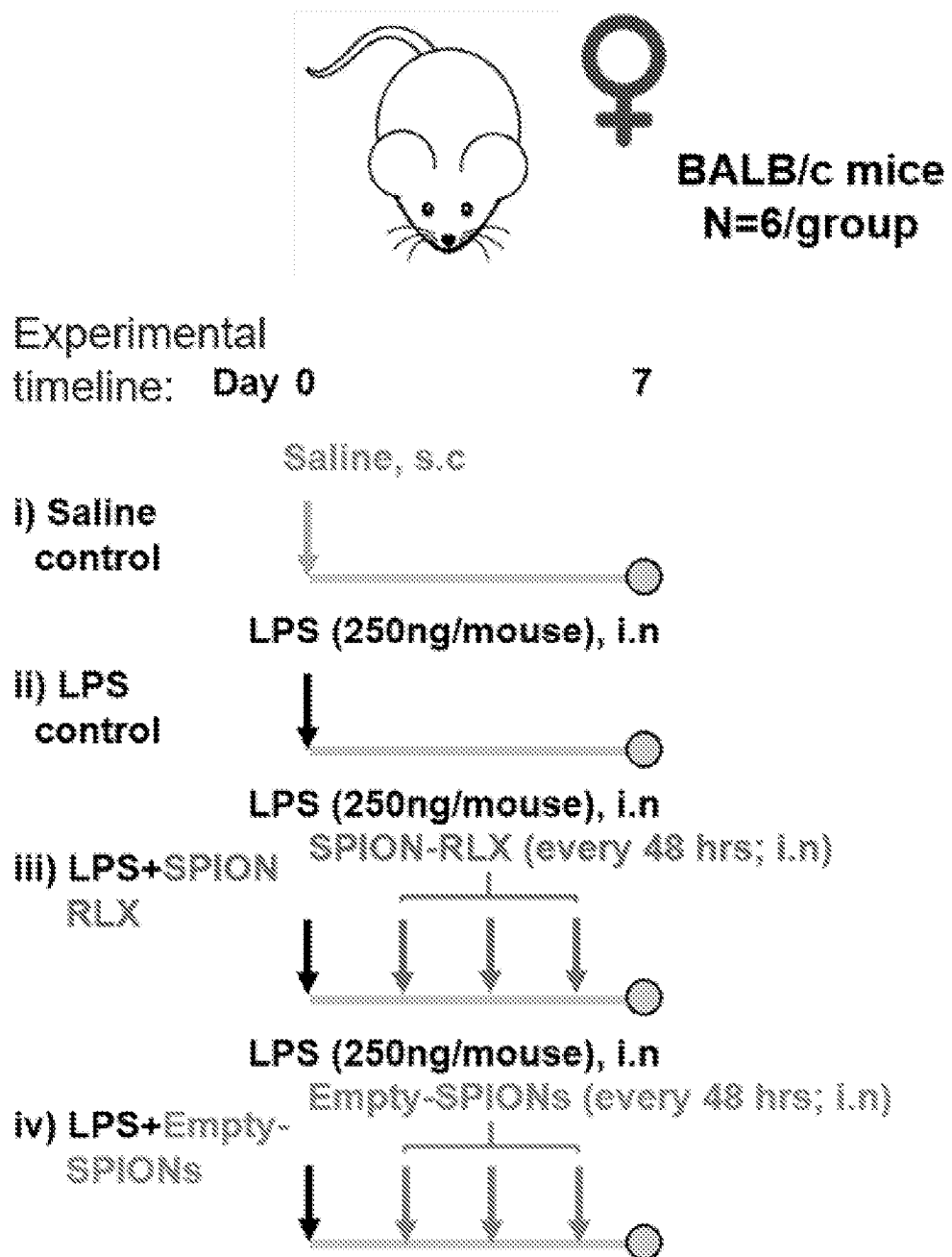


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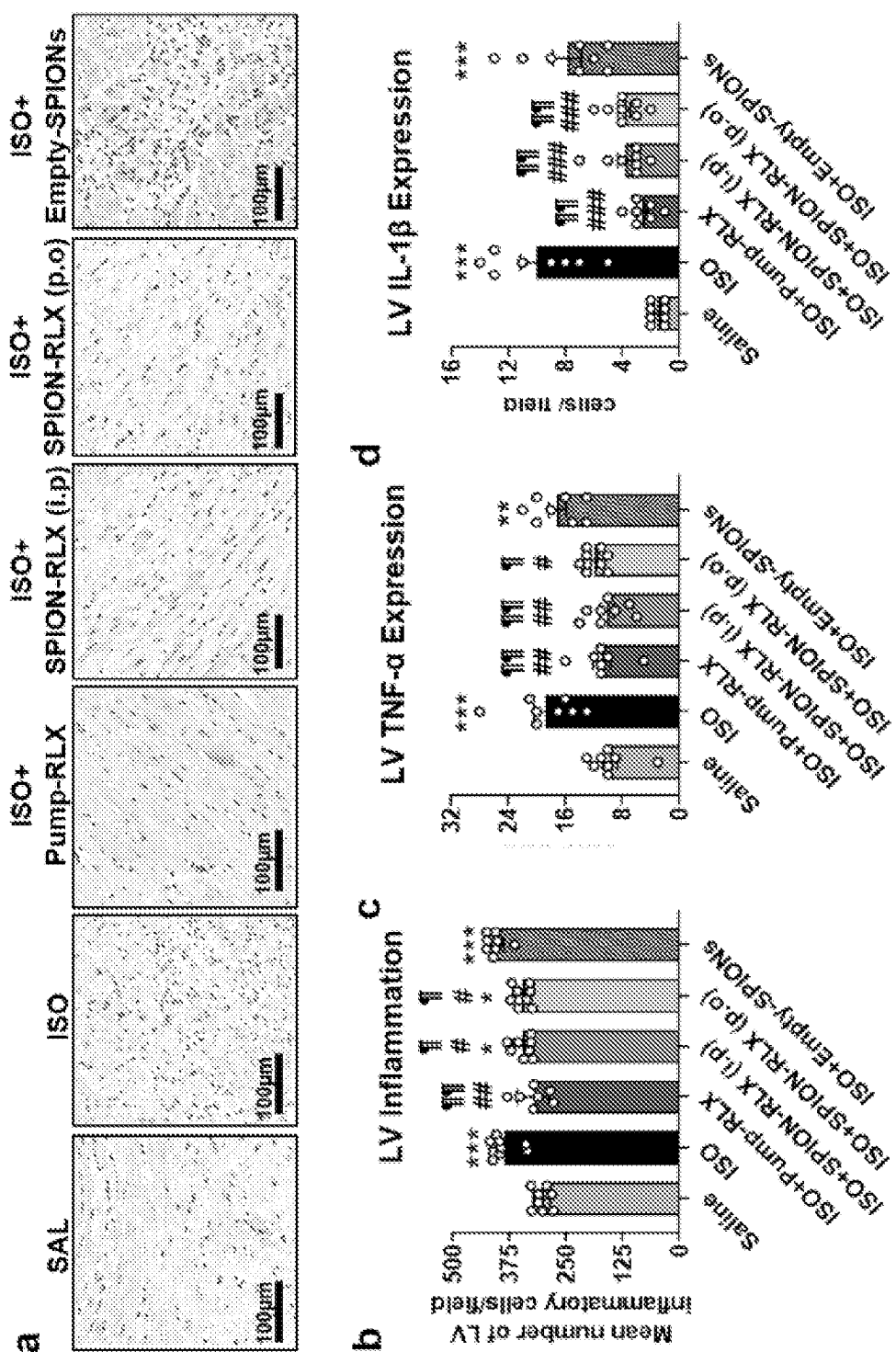
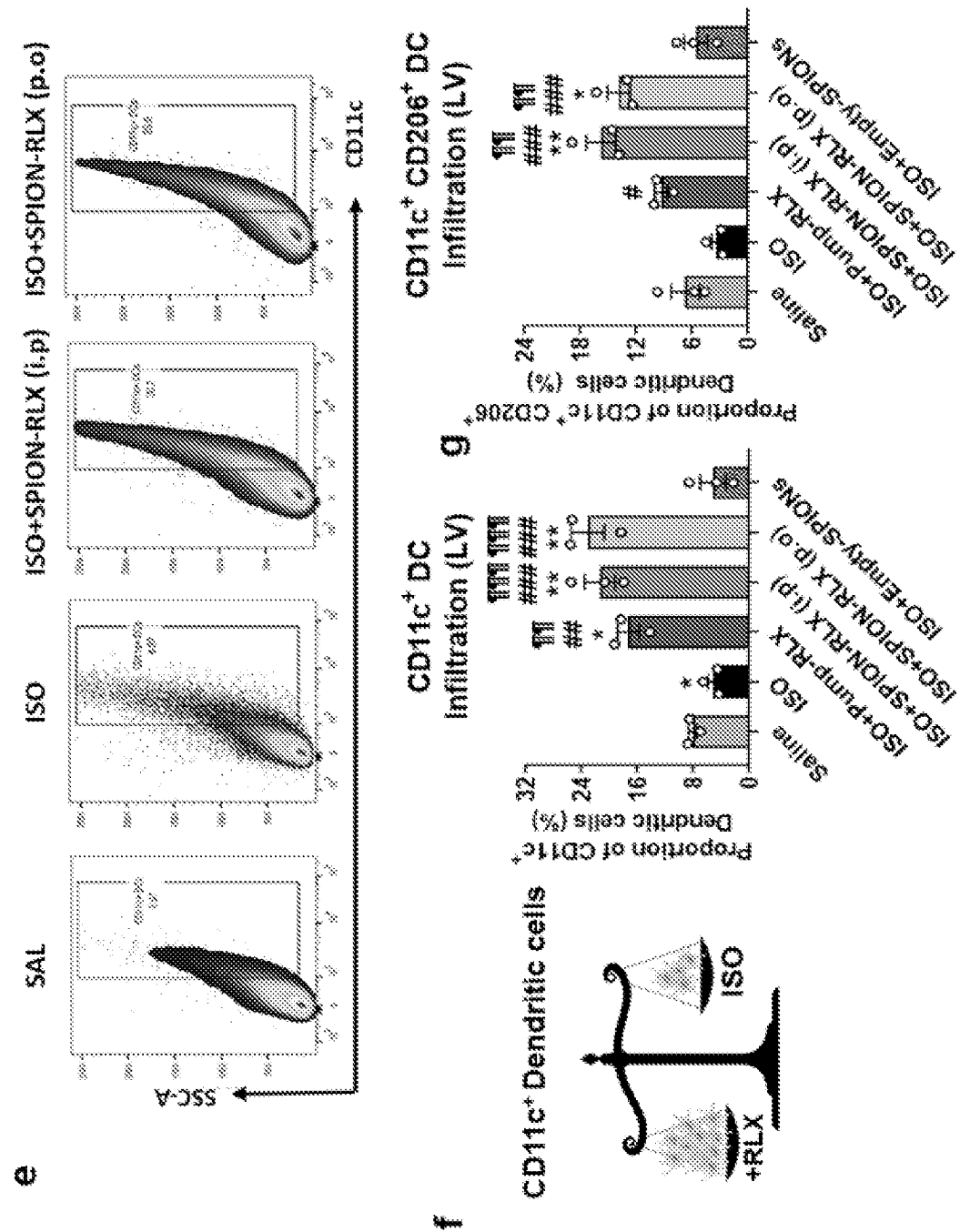


Figure 4 cont.



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Figure 5

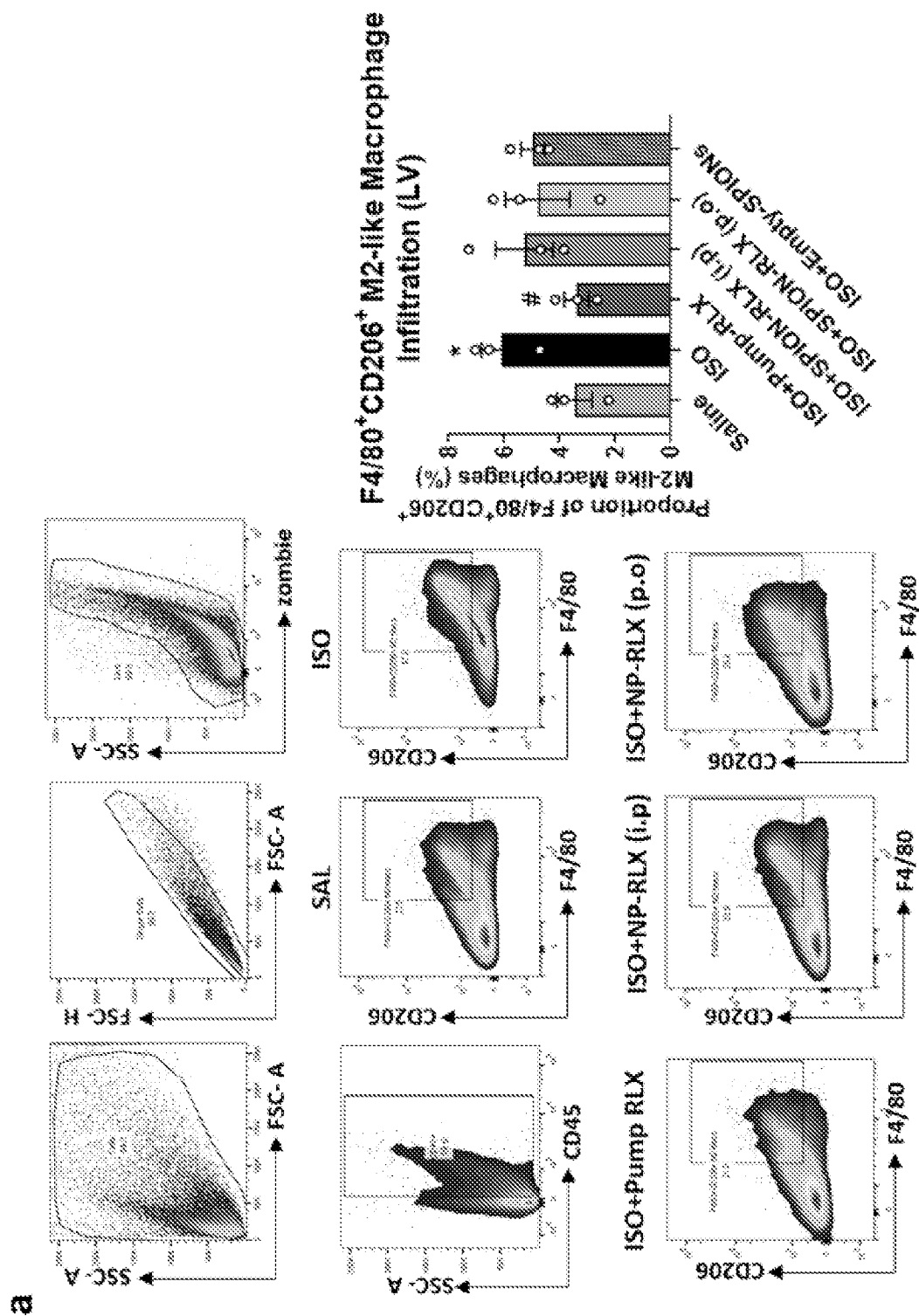
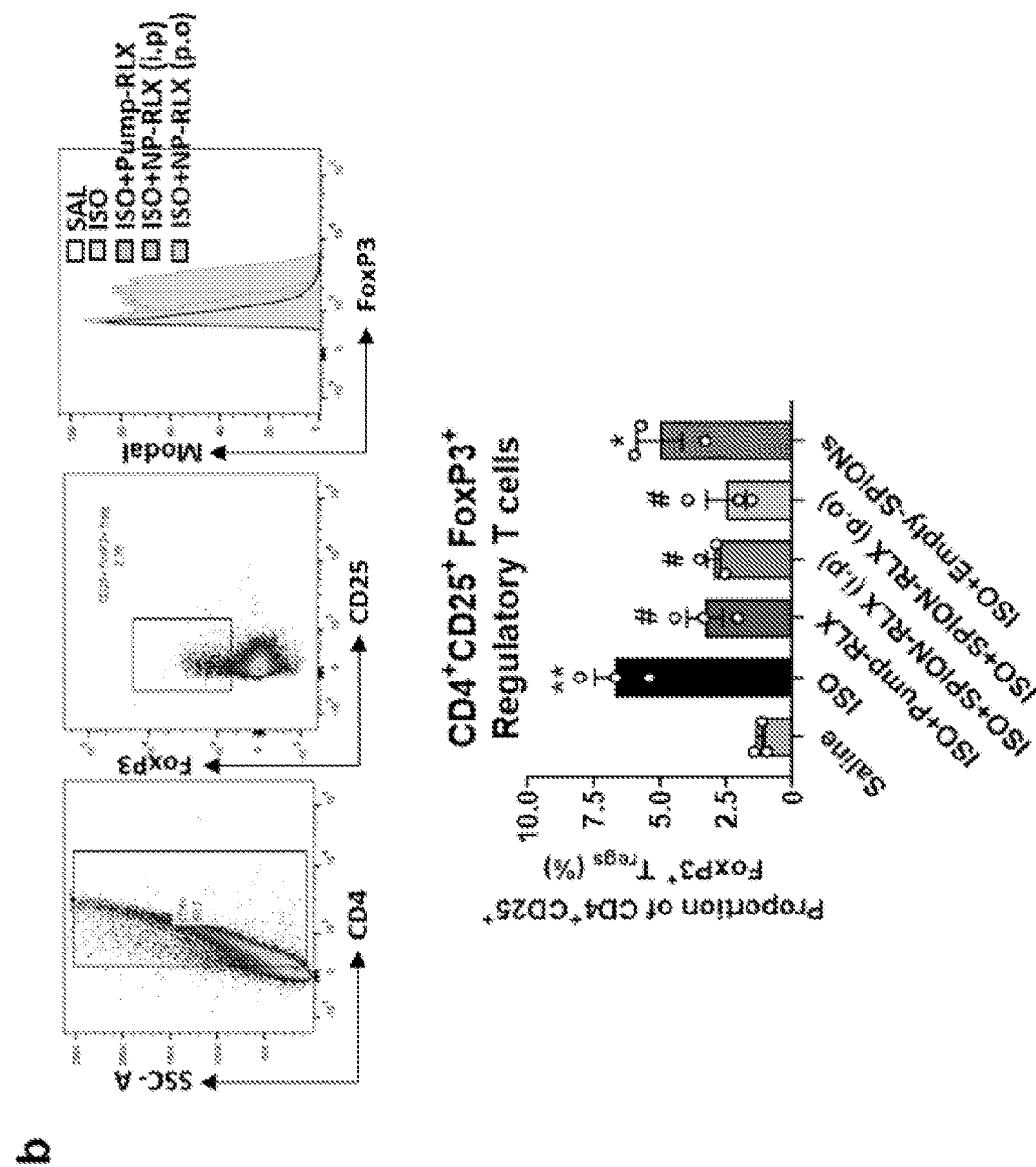
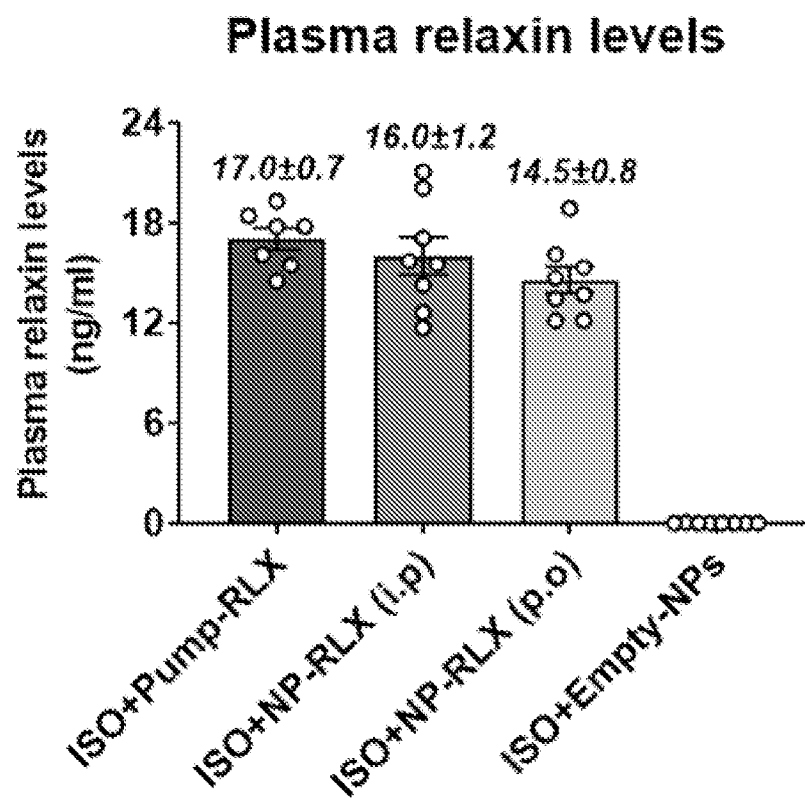


Figure 5 cont.



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Figure 6



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Figure 7

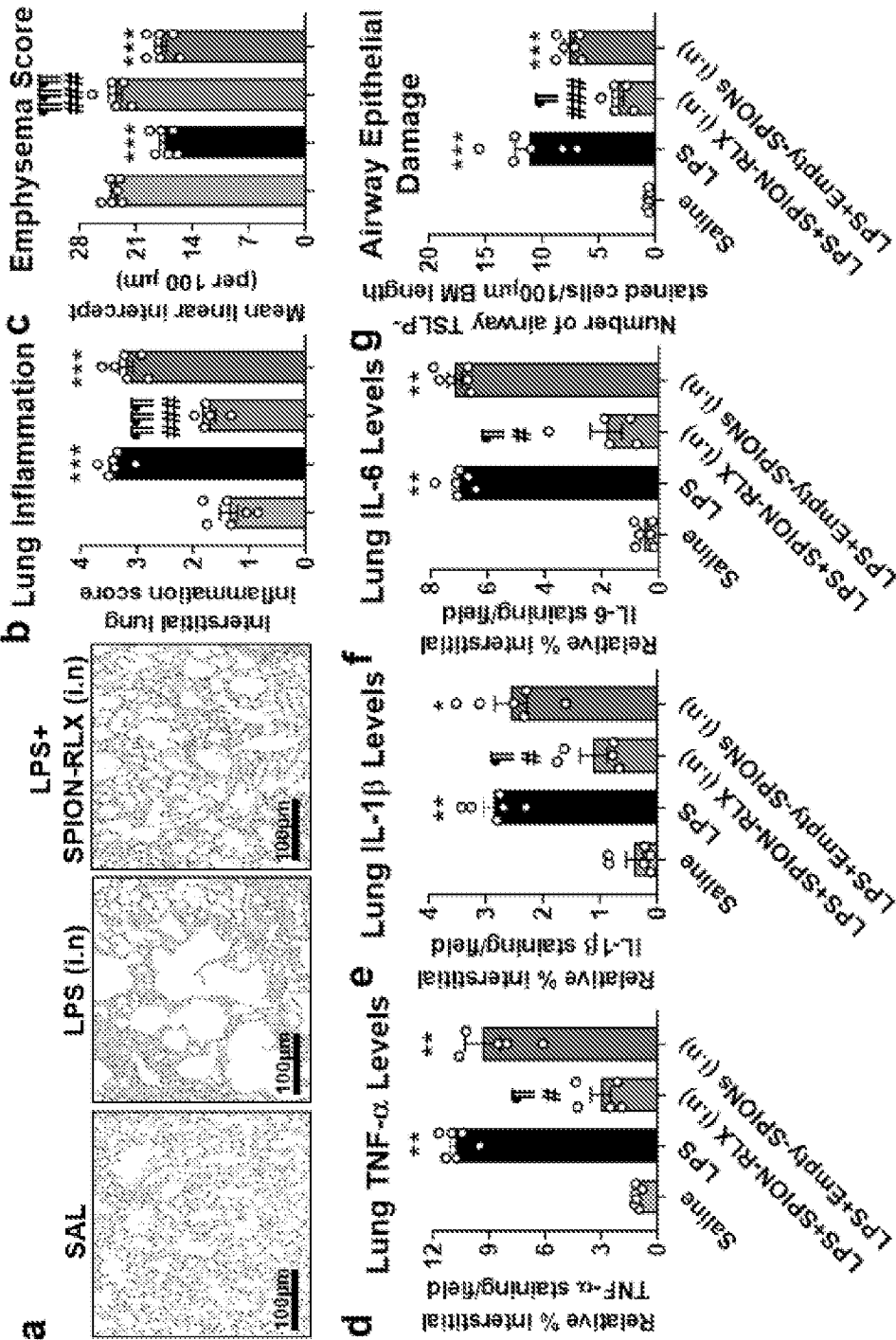


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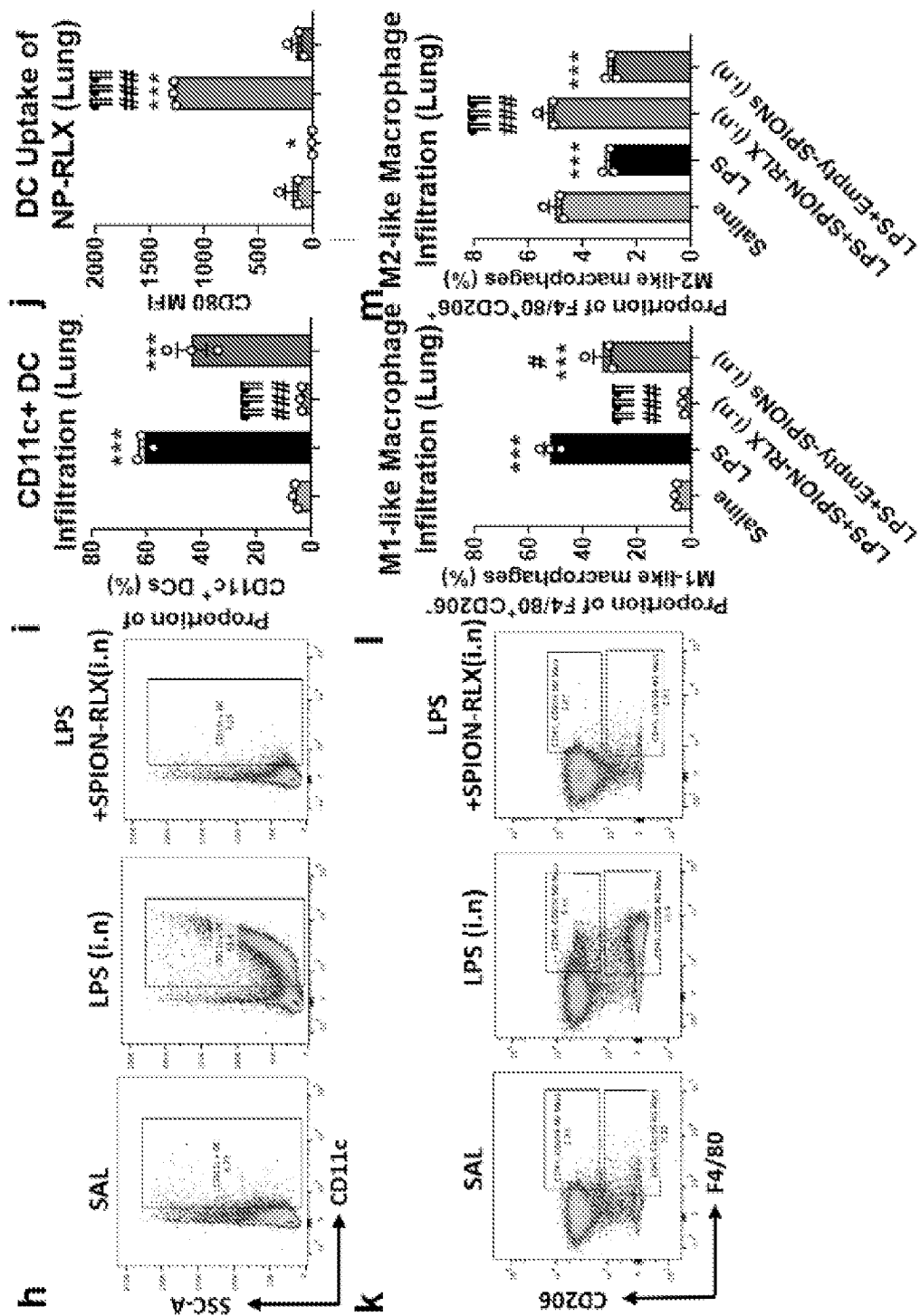
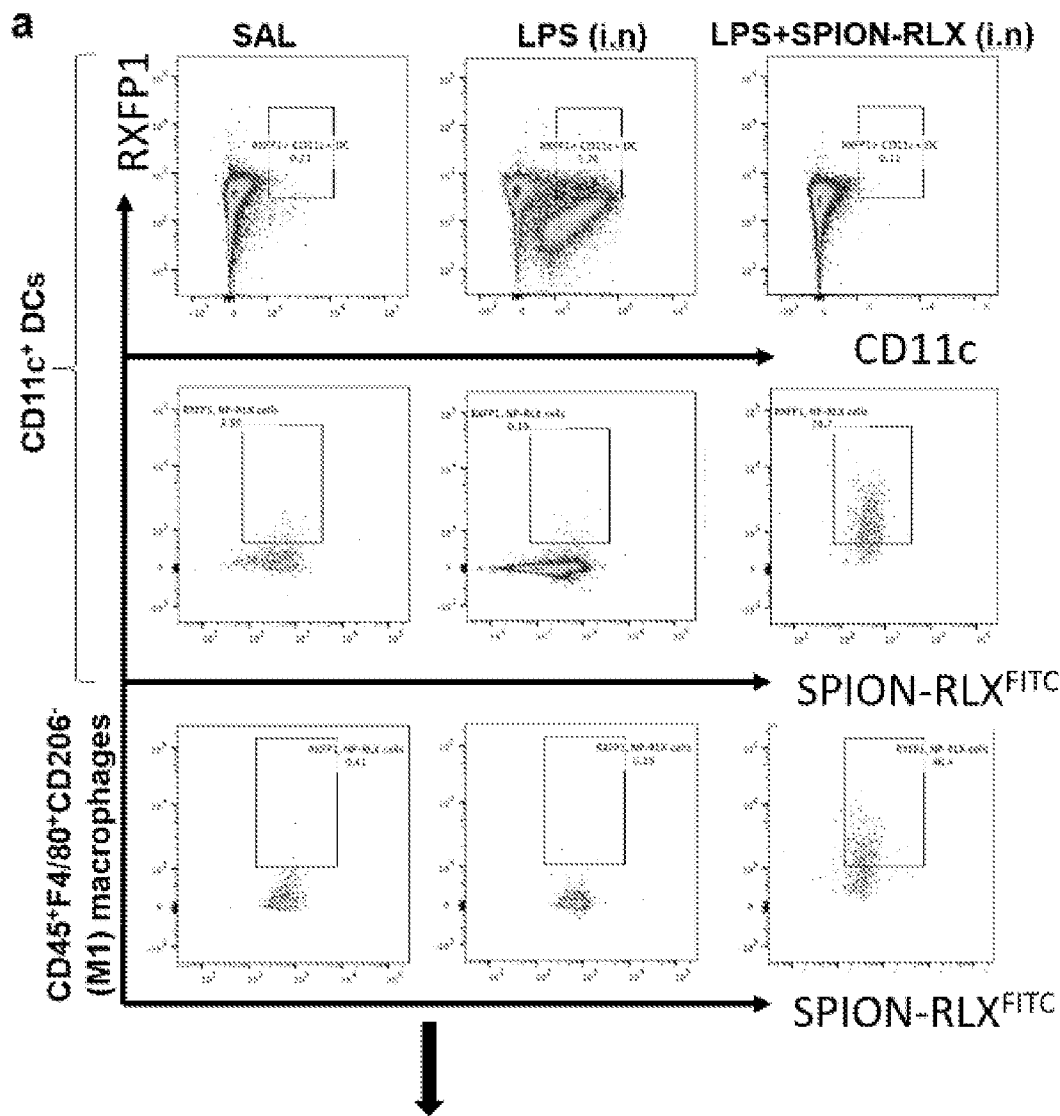


Figure 8



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Figure 8 cont.

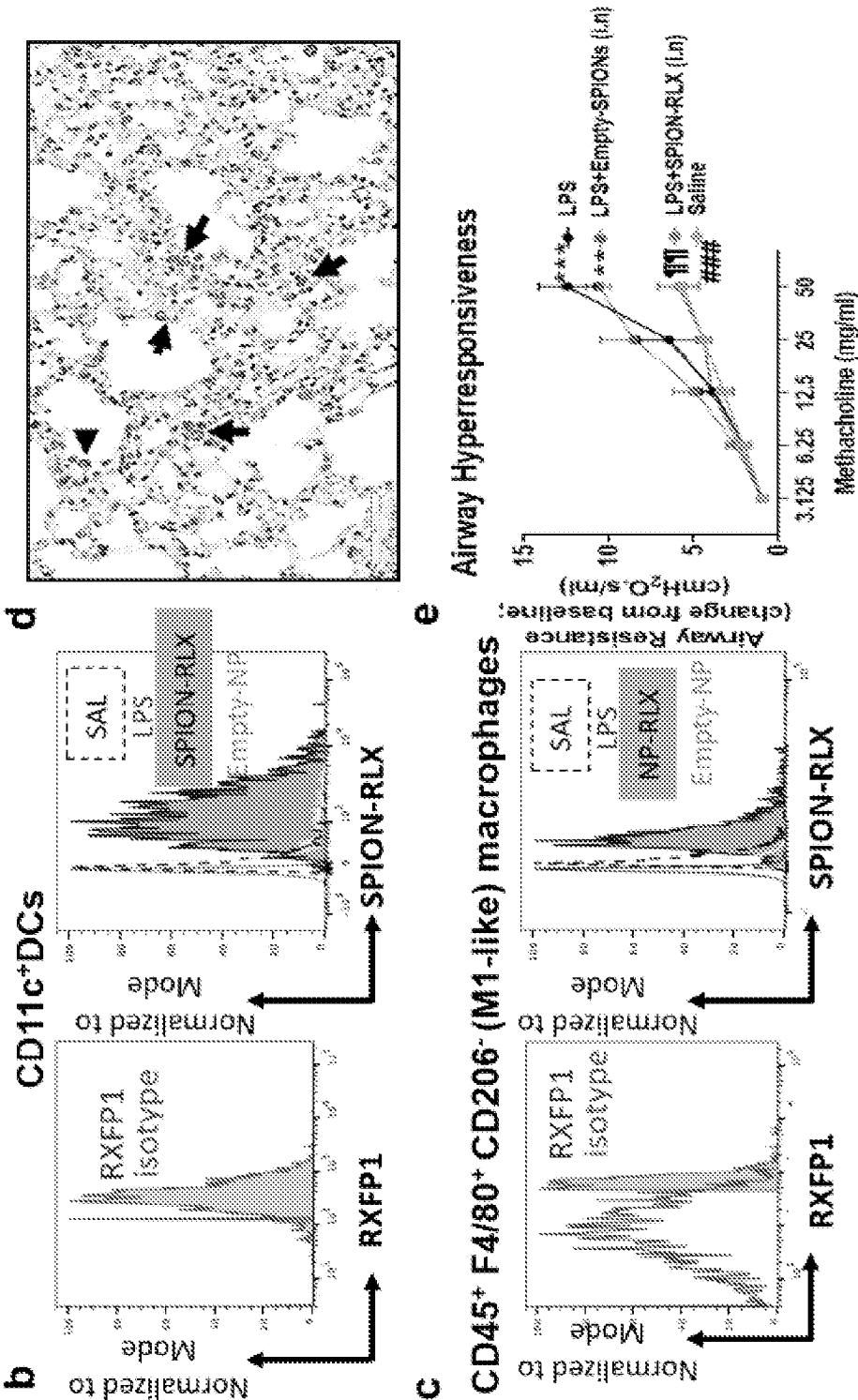


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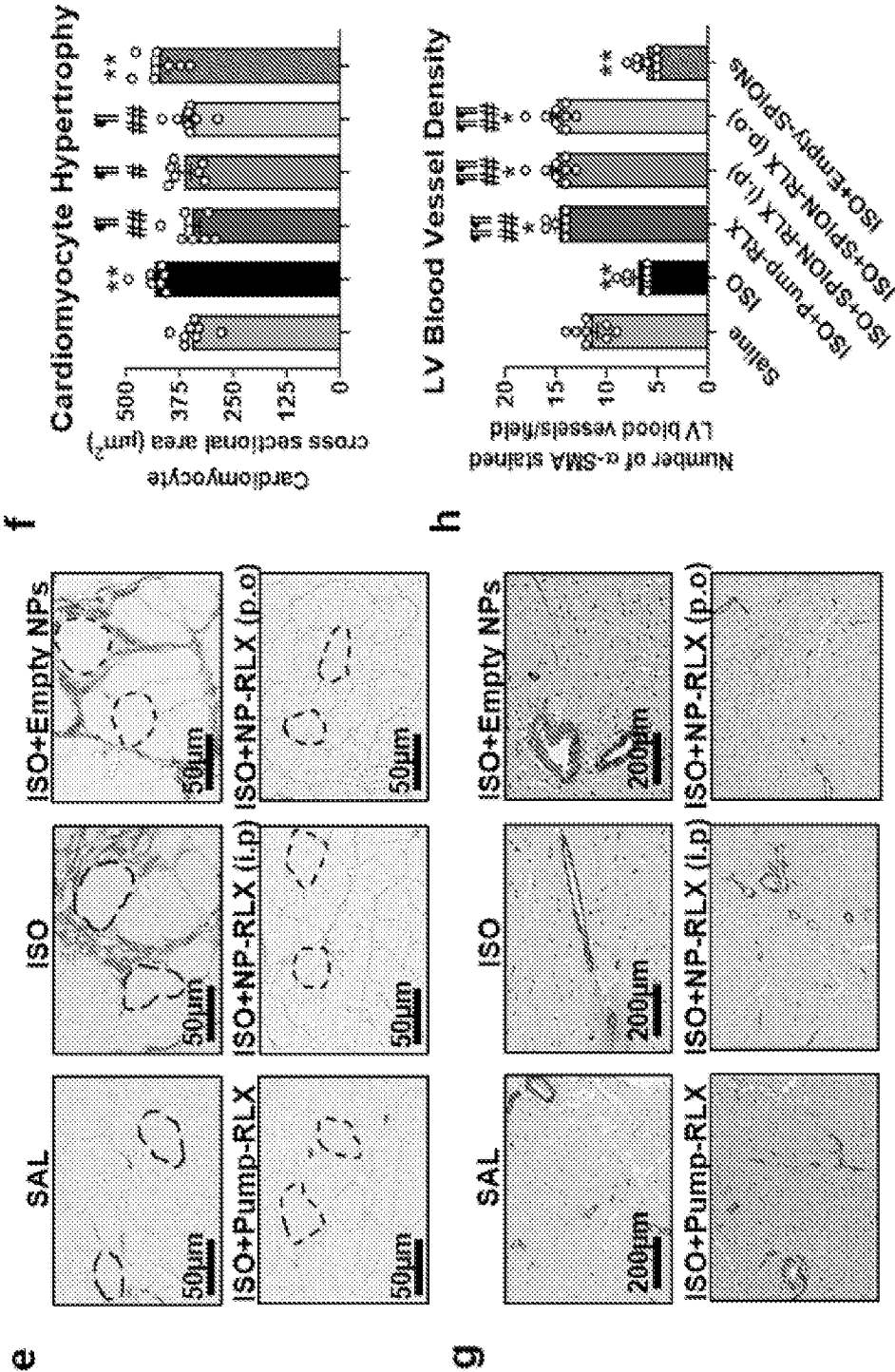
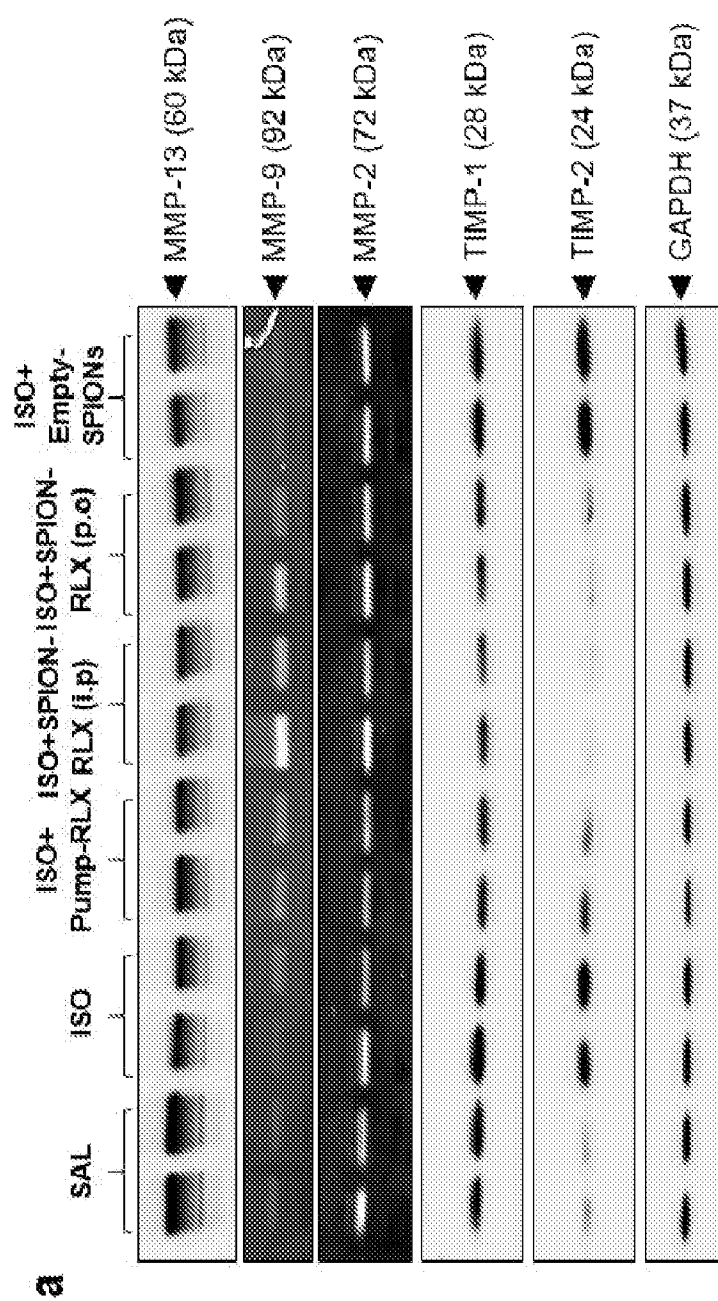


Figure 10



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Figure 10 cont.

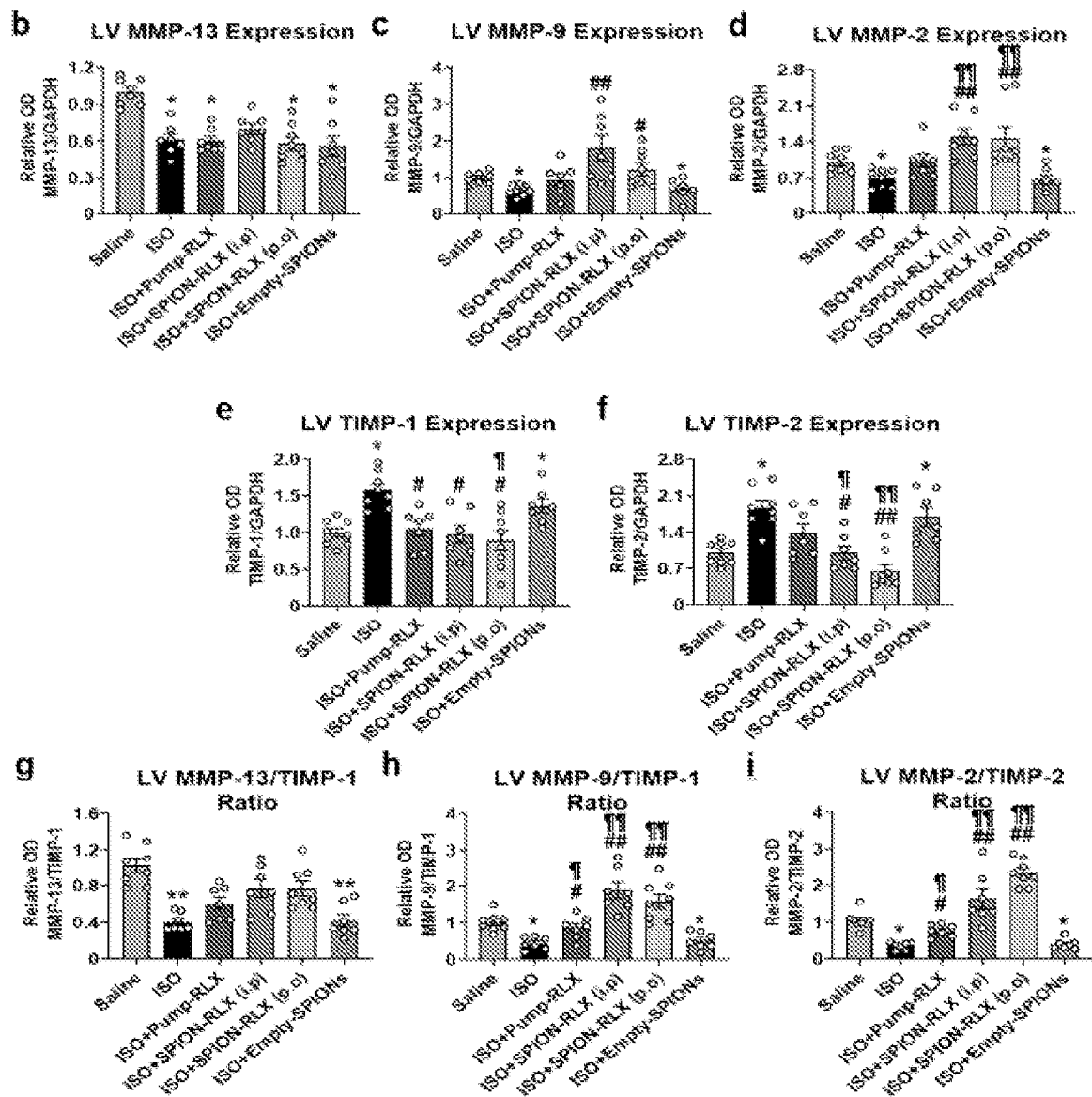


Figure 11

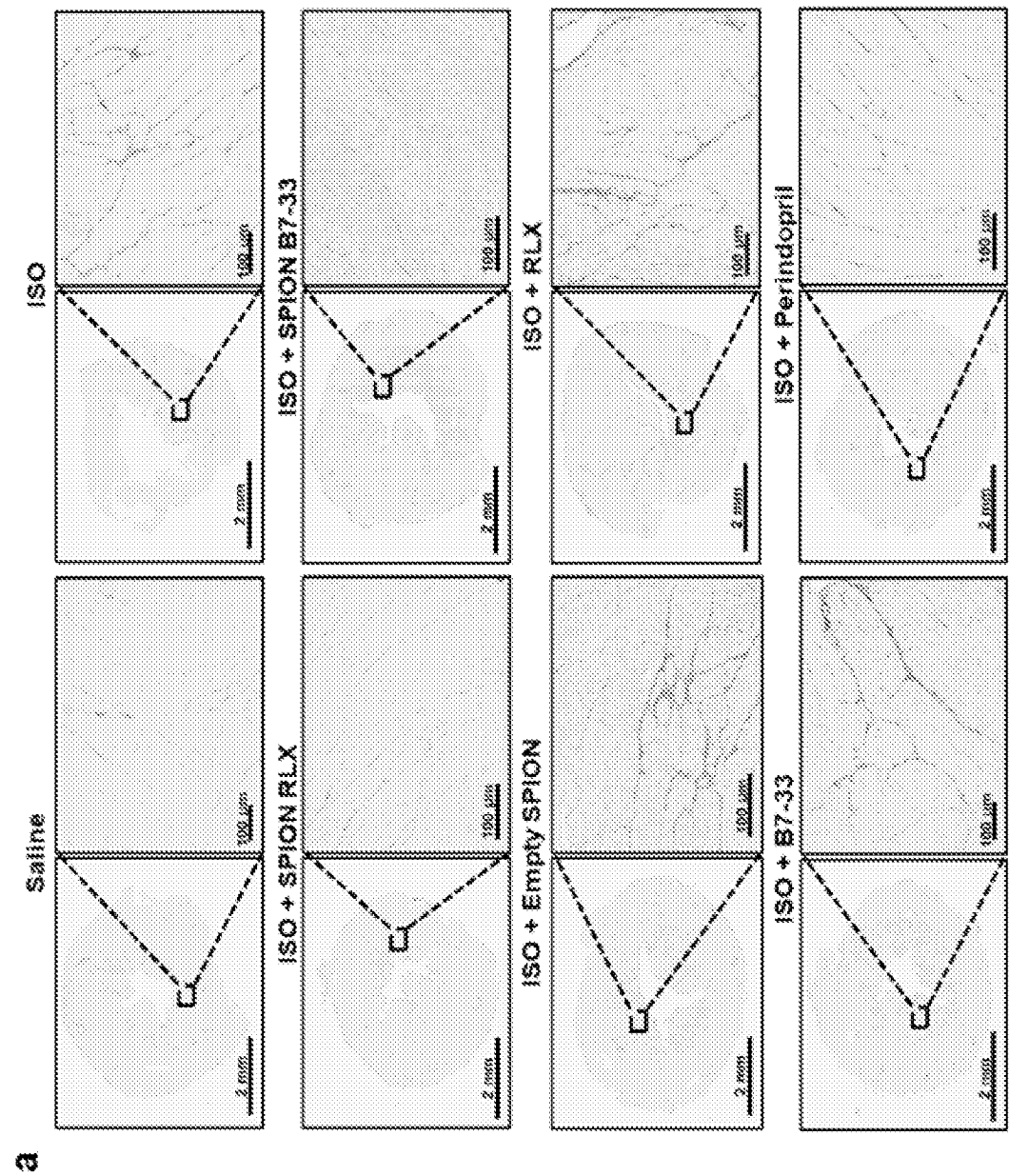


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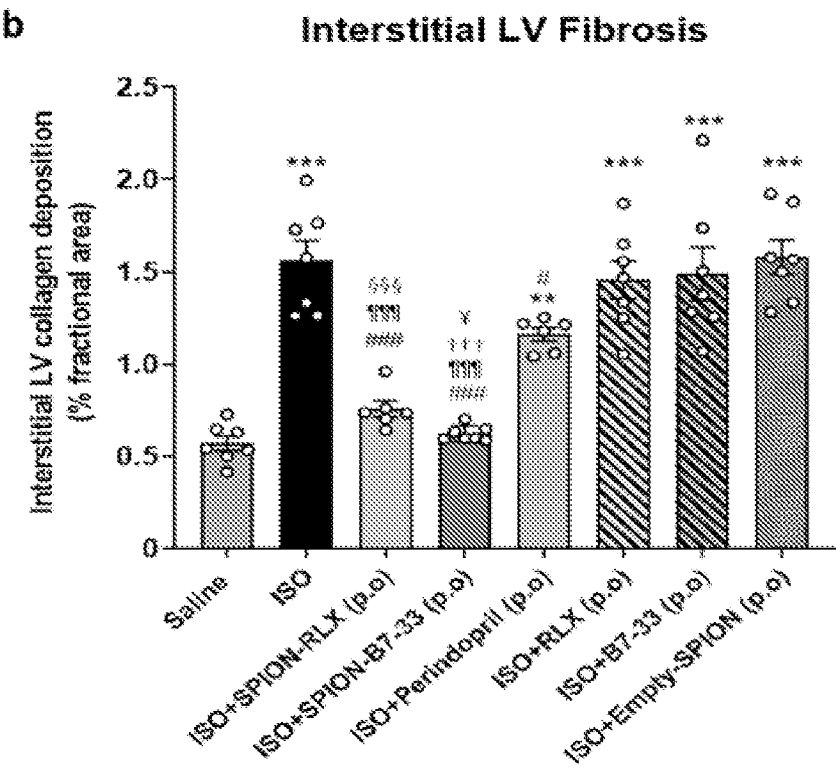
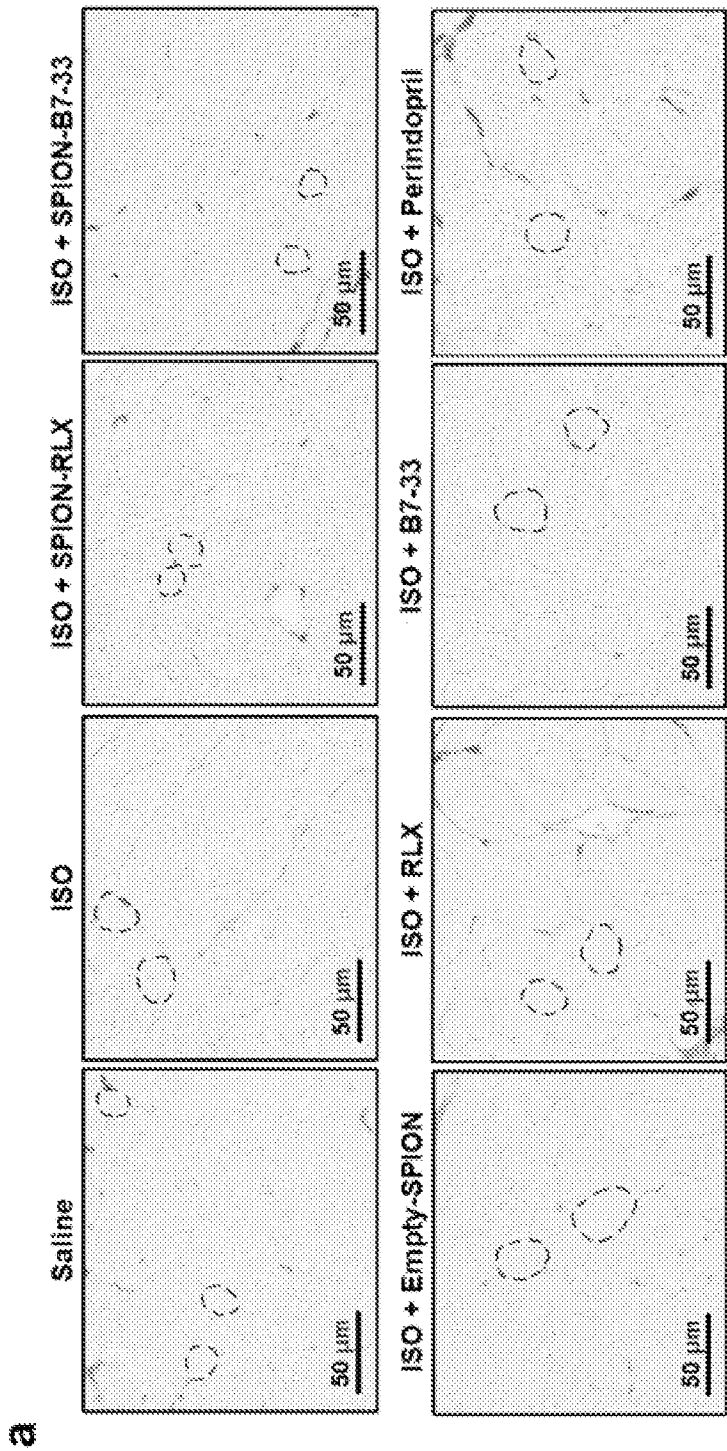


Figure 12



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Figure 12 cont.

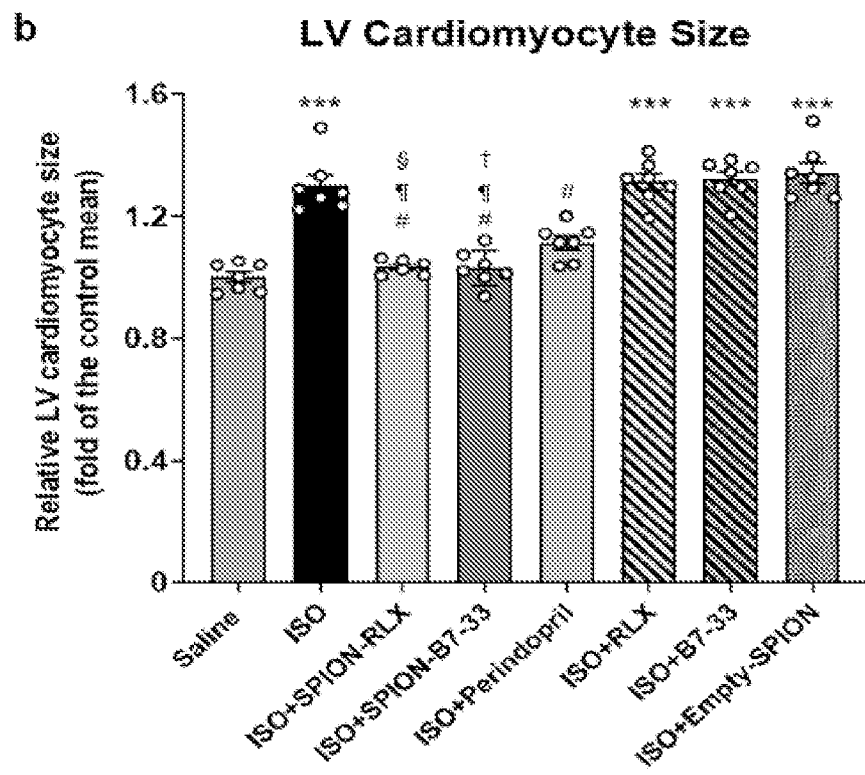
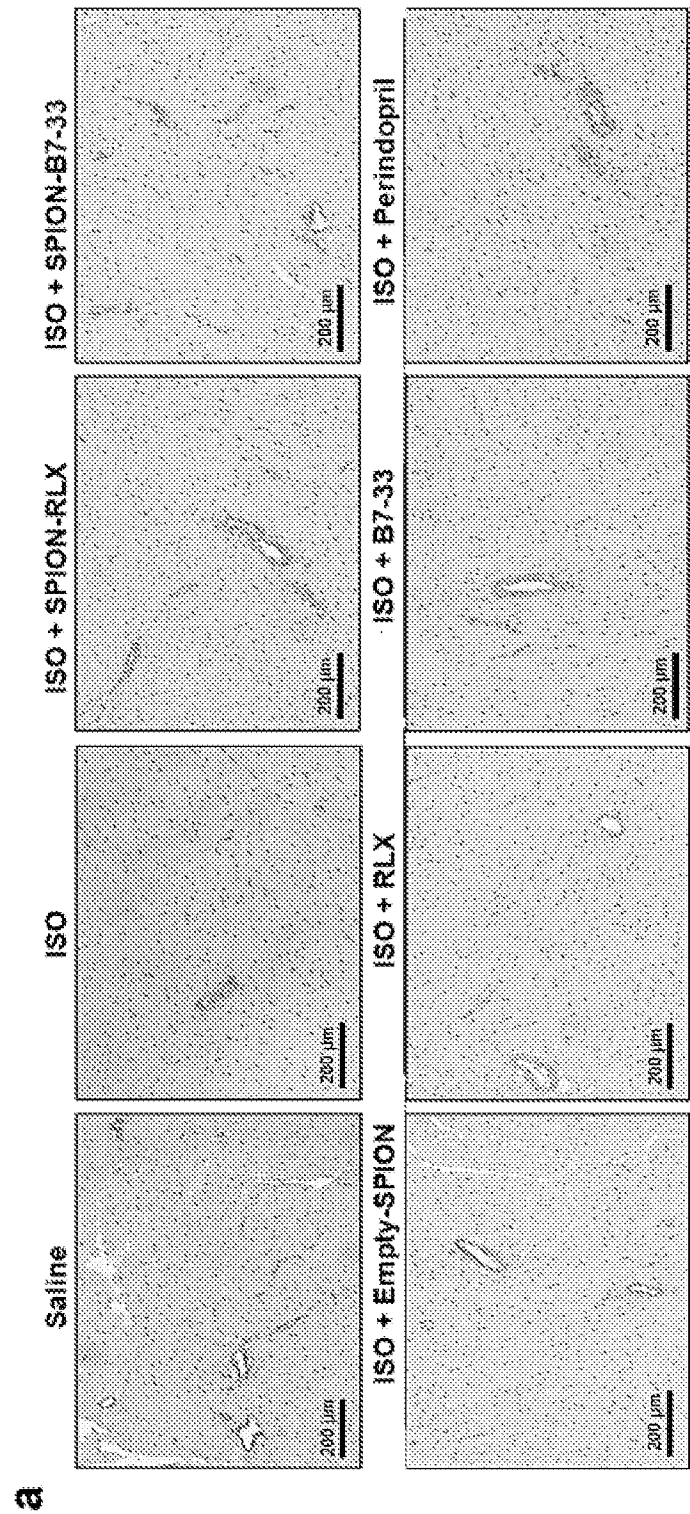
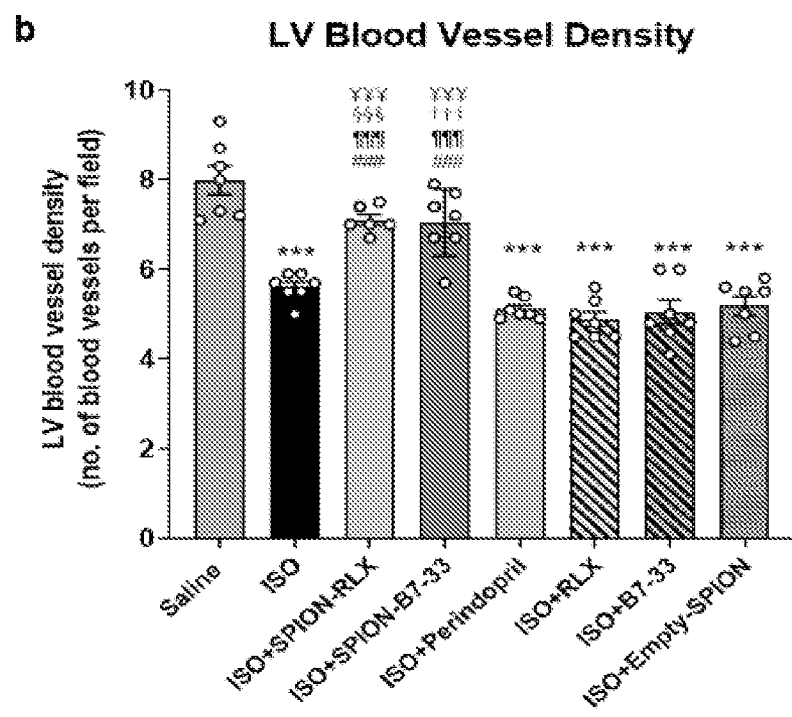


Figure 13



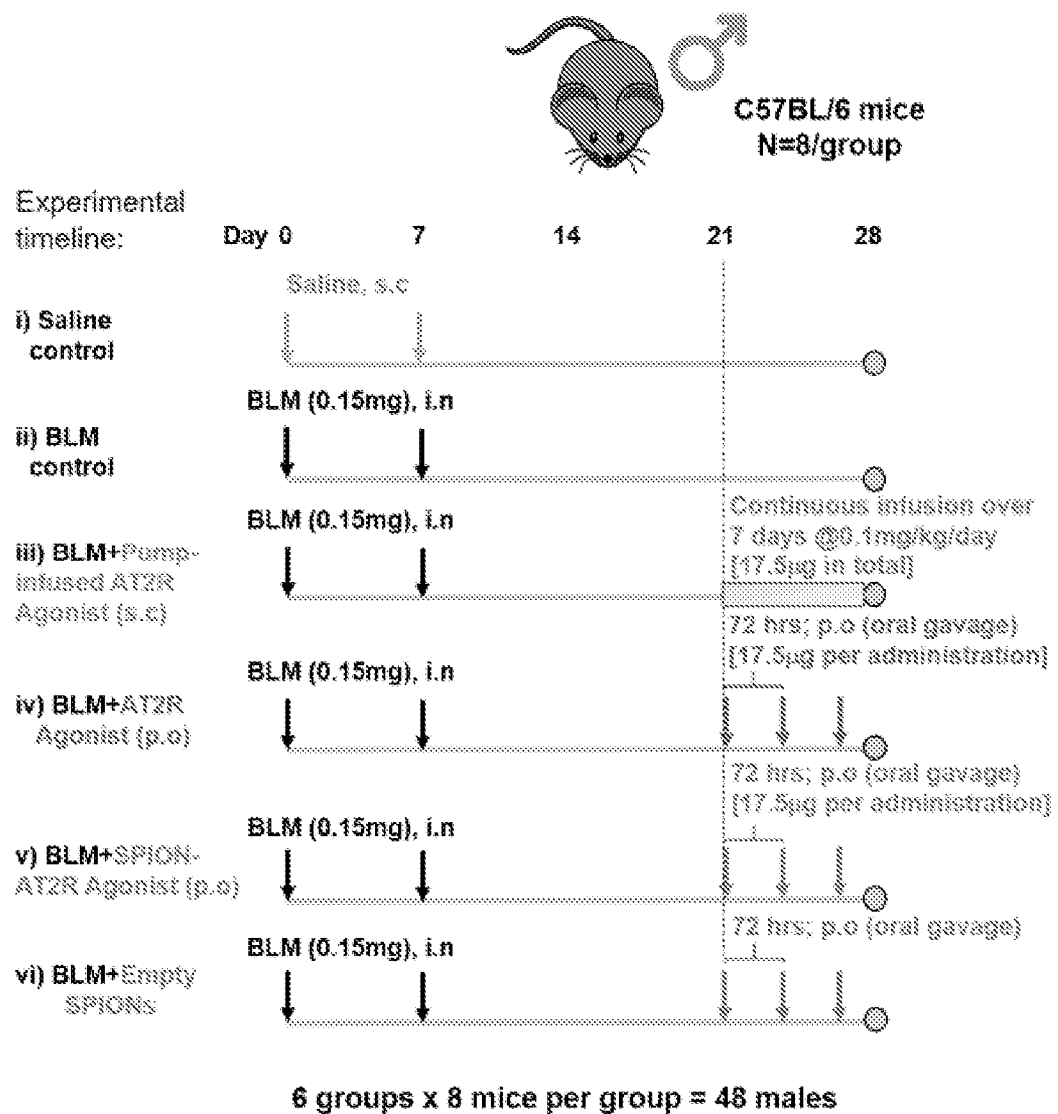
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Figure 13 cont.



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Figure 14



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Figure 15

a

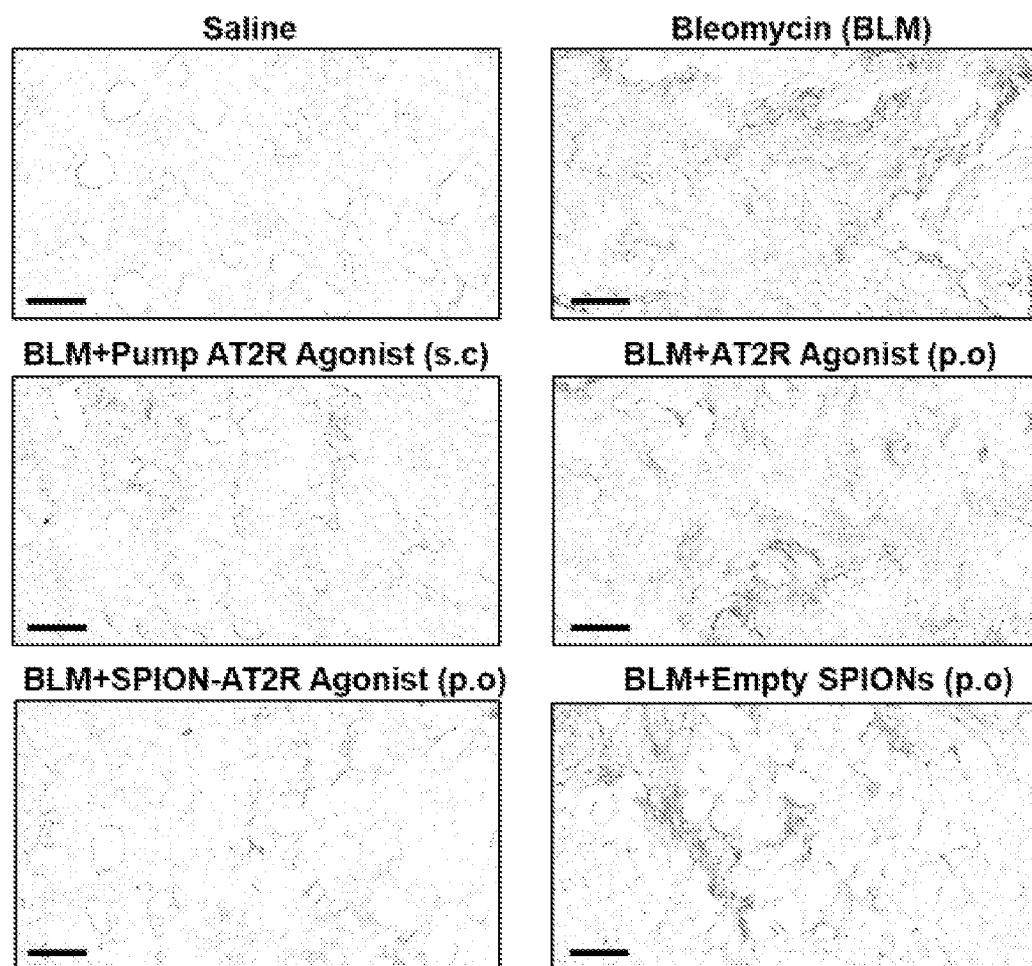
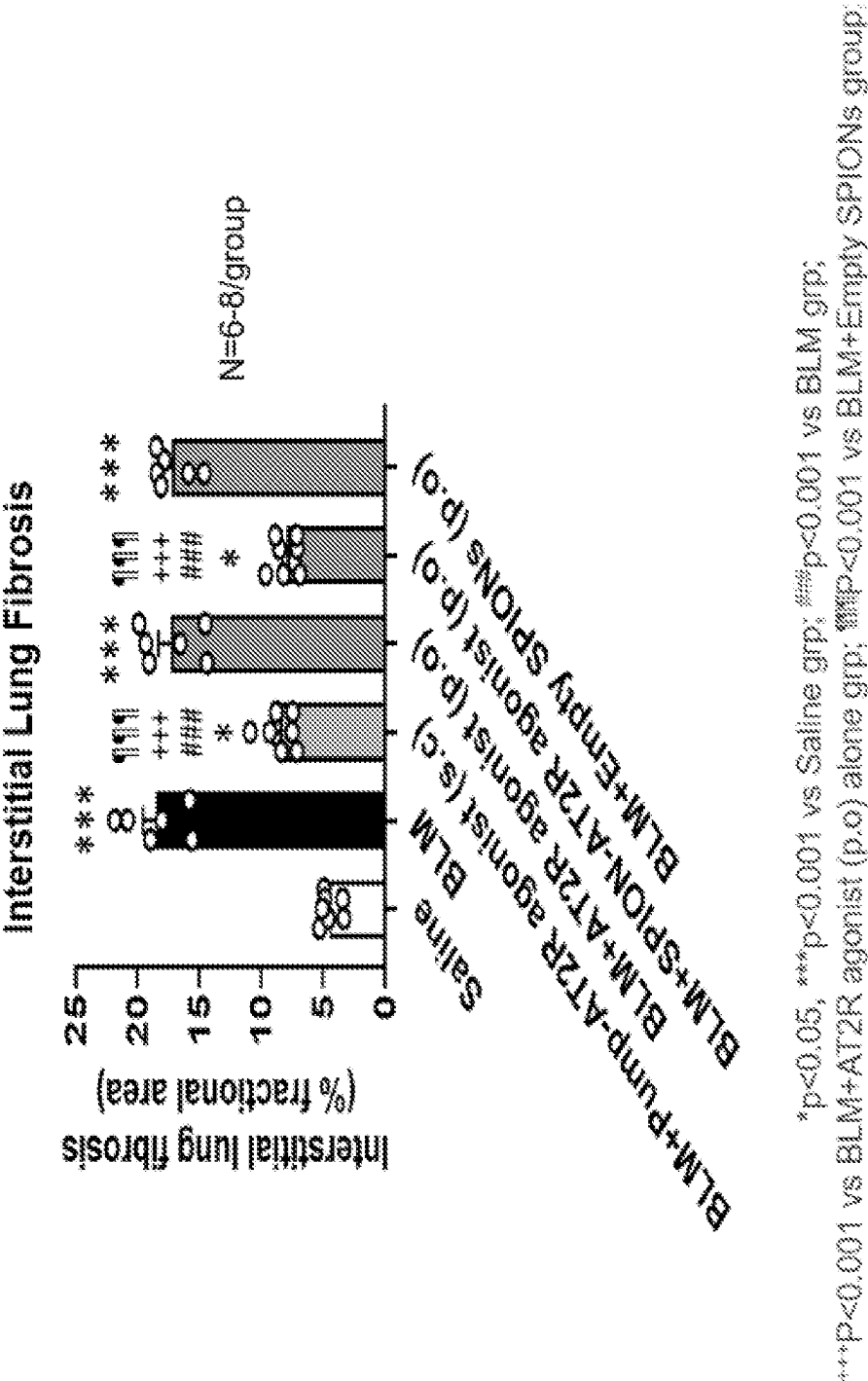


Figure 15 cont.

b



INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2024/050877

A. CLASSIFICATION OF SUBJECT MATTER

A61K 47/52 (2017.01) A61K 9/00 (2006.01) A61K 9/20 (2006.01) A61K 38/08 (2019.01) A61K 38/22 (2006.01)
 A61K 47/18 (2017.01) A61K 47/69 (2017.01) A61K 49/18 (2006.01) A61P 9/00 (2006.01) A61P 11/00 (2006.01)
 A61P 17/02 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

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

EPOQUE: - databases: EPODOC, WPIAP, TXTE; **STN:** databases: BIOSIS, EMBASE, MEDLINE, HCAPLUS; **Internet search engines:** Google, Espacenet, Pubmed.

Keywords: SPION, glycine, peptides, serelaxin, oral administration and like terms.

Applicant/Inventor names searched in DOCDB, DWPI, and Internal Databases.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

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

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
23 September 2024

Date of mailing of the international search report
23 September 2024

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2024/050877
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHAKRABORTY, A. et al., 'Pulmonary myeloid cell uptake of biodegradable nanoparticles conjugated with an anti-fibrotic agent provides a novel strategy for treating chornic allergic airways disease', Biomaterials. 2021, vol. 273, abstract 120796, pages 1-18. https://doi.org/10.1016/j.biomaterials.2021.120796 [Abstract]; [Page 2, paragraph 2]; [Page 2, Section 2.3. Fabrication and characterisation of serelaxin-conjugated SPIONs]; [Page 4, Section 2.6 Induction and treatment of chronic AAD incorporating airway inflammation, airway remodeling and airway hyperactivity]; [Pages 10-12, Section 3.6. NP-RLX reduces established AWR including airway fibrosis in mice with chronic AAD]; [Page 17, Section 5. Conclusions]; [Figure 1]	1-26
A	CHAKRABORTY, A. et al., 'A Novel Approach for Non-Invasive Lung Imaging and Targeting Lung Immune Cells', International Journal of Molecular Sciences. 2020, vol. 21, issue 5, article 1613, pages 1-18. https://doi.org/10.3390/ijms21051613	
A	CHAKRABORTY, A. et al., 'Glycine microparticles loaded with functionalized nanoparticles for pulmonary delivery', International Journal of Pharmaceutics. 2019, vol. 570, article 118654, pages 1-9. https://doi.org/10.1016/j.ijpharm.2019.118654	
A	WO 2013/127829 A1 (UNIVERSITAT DE BARCELONA et al.) 06 September 2013 [Example 11]	
A	WO 2018/064150 A1 (THE UNIVERSITY OF MEMPHIS RESEARCH FOUNDATION) 05 April 2018 [Page 41, paragraph 2]	
A	CHAKRABORTY, A. et al., 'Amino Acid Functionalized Inorganic Nanoparticles as Cutting-Edge Therapeutic and Diagnostic Agents', Bioconjugate Chemistry. 2018, vol. 29, issue 3, pages 657-671. https://doi.org/10.1021/acs.bioconjchem.7b00455	
Form PCT/ISA/210 (fifth sheet) (July 2019)		

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2024/050877	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2013/127829 A1	06 September 2013	WO 2013127829 A1	06 Sep 2013
		EP 2819700 A1	07 Jan 2015
		EP 2819700 B1	21 Dec 2016
		US 2015044140 A1	12 Feb 2015
		US 9475840 B2	25 Oct 2016
WO 2018/064150 A1	05 April 2018	WO 2018064150 A1	05 Apr 2018
		US 2021275440 A1	09 Sep 2021
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			
Form PCT/ISA/210 (Family Annex)(July 2019)			