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(71) Applicant: UNIVERSITY OF VIRGINIA PATENT
FOUNDATION [US/US]; 722 Preston Avenue, Suite 107,
P.O. Box 800755, Charlottesville, Virginia 22903 (US).

(72) Inventors: SUN, Jie; 360 Bishopgate Lane, Crozet, Vir-
ginia 22932 (US). WEI, Xiaoqin; 2512 Fontaine Avenue,
Apartment B, Charlottesville, Virginia 22903 (US).

(74) Agent: ROMAGNANO, Joseph F.; Cantor Colburn LLP,
20 Church Street, 22nd Floor, Hartford, Connecticut
06103-3207 (US).

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(54) Title: METHODS OF PROMOTING LUNG HEALING AND LUNG TISSUE REGENERATION FOLLOWING INJURY OR
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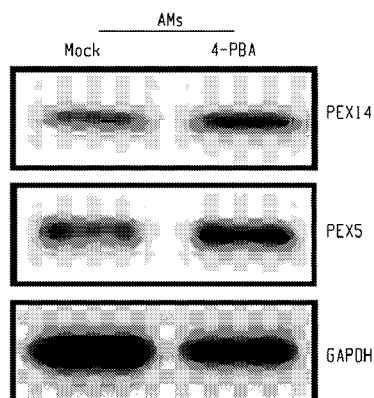
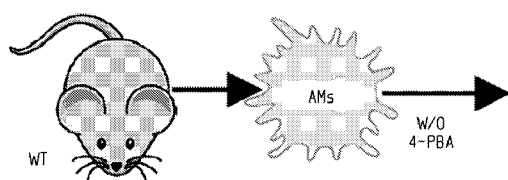


Fig. 10

(57) Abstract: Disclosed is a method of treating lung injury in a patient comprising providing a therapeutically effective amount of a pharmaceutical agent that enhances peroxisome biogenesis to a patient. The method includes increasing the number of peroxisomes in alveolar macrophages, decreasing peroxisome degradation, and improving peroxisome function in alveolar macrophages. Increasing the number and function of peroxisomes in alveolar macrophages promotes healing of lung injury and regeneration of alveolar epithelial. The lung injury may be due to infection, including viral infection, such as SARS-CoV-2 infection or influenza infection. The lung injury may also be due to exposure to caustic substances, tobacco smoke, asbestos, or fine particulate matter.

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- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
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METHODS OF PROMOTING LUNG HEALING AND LUNG TISSUE REGENERATION FOLLOWING INJURY OR VIRAL INFECTION

GOVERNMENT SUPPORT

[0001] This invention was made in part with government support under AG069264 awarded by the National Institutes of Health. The US government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims the benefit of U.S. Prov. Pat. App. No: 63/610,838 filed 15 December 2023, the entirety of which is hereby incorporated by reference.

BACKGROUND

[0003] The COVID-19 pandemic, caused by coronavirus SARS-CoV-2, and the multiple variants thereof, posed a tremendous threat to human health and public safety with manifestations of infection ranging from severe acute symptoms to lingering chronic complications. Global efforts first concentrated on treating the acute symptoms of the virus. Emerging evidence reveals widespread prevalence of long-term pulmonary and extrapulmonary complications termed post-acute sequelae of SARS-CoV-2 infection (PASC). Persistent chronic sequelae are further observed in the context of other respiratory viral infections, including: influenza virus, respiratory syncytial virus, rhinovirus, severe acute respiratory syndrome coronavirus, and Middle Eastern respiratory syndrome coronavirus. At present, the intricate molecular and metabolic mechanisms driving both the acute disease presentation and the chronic symptoms after respiratory viral infections remain largely unidentified. In the case of COVID-19, antiviral (*e.g.*, paxlovid and molnupiravir) and anti-inflammatory (*e.g.*, dexamethasone and tocilizumab) treatment strategies improved prognosis and outcomes in the acute phase of the disease. The immense healthcare burden presented by PASC establishes an urgent need for development of pro-repair therapeutics, aimed towards the enhancement of lung regeneration and host recovery after acute SARS-CoV-2, or other respiratory virus, mediated injury.

SUMMARY

[0004] This disclosure addresses the urgent need for lung repair therapeutics. It provides methods of pharmacologically enhancing peroxisome biogenesis; thereby augmenting lung

regeneration, dampening acute diseases, and alleviating chronic sequelae post viral infection. This disclosure also provides methods of treating lung damage due to respiratory viral infections and a peroxisome-centric therapeutic strategy to manage both acute and long-term consequences of COVID-19.

[0005] This disclosure includes a method of treating lung injury in a patient or subject in need thereof. Embodiments of the method comprise providing a therapeutically effective amount of a pharmaceutical agent that enhances peroxisome biogenesis to a patient. The method includes increasing the number of peroxisomes in alveolar macrophages, decreasing peroxisome degradation, and improving peroxisome function in alveolar macrophages. Increasing the number and function of peroxisomes in alveolar macrophages promotes healing of lung injury and regeneration of alveolar epithelial. The lung injury may be due to infection, including viral infection, such as Sars-CoV-2 infection or influenza infection. The lung injury may also be due to exposure to caustic substances, tobacco smoke, asbestos, or fine particulate matter.

[0006] This disclosure also provides a method of treating brain injury due to viral infection, liver injury due to viral infection, or liver damage due to fatty liver or alcoholism, comprising providing a therapeutically effective amount of a pharmaceutical agent that enhances peroxisome biogenesis to a patient.

[0007] The disclosure includes a method of treating long COVID or preventing long COVID in a patient comprising providing a therapeutically effective amount of a pharmaceutical agent that enhances peroxisome biogenesis to the patient, where the patient is a patient that is infected with a Sars-CoV-2 virus or has been infected with a Sars-CoV-2 virus within the past 2 weeks to 6 months. The patient can be a patient that has an active Sars-CoV-2 infection or a patient that is no longer experiencing symptoms of Sars-CoV-2 active infection. In certain embodiments the symptoms no longer experienced are acute symptoms while chronic symptoms may still be present.

[0008] The disclosure includes a method of promoting lung healing and lung tissue regeneration, comprising providing a pharmaceutical agent that enhances peroxisome biogenesis to the patient.

[0009] The pharmaceutical agent can be 4-phenylbutyrate, a monounsaturated fatty acid, such as an omega-3 fatty acid (*e.g.* DHA or EPA) or an omega-6 fatty acid, or an alpha, beta, or gamma PPAR (peroxisome proliferator-activated receptor) agonist. The PPAR agonist can be clofibrate, gemfibrozil, ciprofibrate bezafibrate, fenofibrate, pioglitazone, rosiglitazone, or lobeglitazone, or any other suitable PPAR agonist.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] FIGURE 1. divided as Figs. 1A-1D presents reduced peroxisome protein expression in lung macrophages after severe viral infection. Fig. 1A, Immunoblot analysis of indicated peroxisomal proteins in control non-COVID (n=4) and acute COVID-19 autopsy (n=4) lung cells. Fig. 1B, Quantification of indicated peroxisomal proteins expression in Fig. 1A. Fig. 1C, Immunoblot analysis of indicated peroxisomal proteins in AMs from naive (n=3) and IAV infected (n=3) C57BL/6 mice at 8 d.p.i.. Fig. 1D, Quantification of indicated peroxisomal proteins expression in IAV infected C57BL/6 mice (n=3-6). Data are representative of at least two independent experiments with similar results. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

[0011] FIGURE 2. divided as Figs. 2A-2D presents how severe acute COVID-19 leads to peroxisome compartment remodeling in macrophages. Fig. 2A, Quantification of PEX14 staining density and % PEX14⁺ cells in total CD68⁺ cells in whole lung sections (n=5-8). Fig. 2B, Quantification of PEX14 staining density and % PEX14⁺ cells in total CD169⁺ cells, and the average size of PEX14 staining structure in samples of 40-50 cells each from COVID-19 autopsy samples. Fig. 2C, % PMP70⁺ AMs in whole slides (n=5), and the size of PMP70 staining structure in 30 cells from SARS-CoV-2 MA30 infected mice. Fig. 2D, % PMP70⁺ AMs in whole slides (n=5), and the size of PMP70 staining structure in 30 cells from IAV infected mice. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.

[0012] FIGURE 3. divided as Figs. 3A-3C presents how interferons inhibit peroxisome biogenesis and promote peroxisome degradation in lung macrophages. Fig. 3A, Representative Western blot for peroxisome proteins, PEX5, PEX14 and PMP70, in primary mouse AMs stimulated with indicated stimulators for 24 h *in vitro*. Fig. 3B, Peroxisome gene expression in mouse AMs stimulated with or without IFN α or IFN γ . Fig. 3C, Diagram depicting a model illustrating how interferons inhibit peroxisome biogenesis and promoting pexophagy. Data are representative of at least two independent experiments with similar results.

[0013] FIGURE 4. divided as Figs. 4A-4J illustrates that macrophage peroxisomes are required for alveolar repair after viral injury. Fig. 4A& Fig. 4B, *Pex5*^{fl/fl} (Pex5 wild type) or *Pex5* ^{Δ Cd11c} (Pex5 deficiency in alveolar macrophages) mice were infected sublethal (Fig. 4A, Fig. 4C, Fig. 4D) and lethal (Fig. 4B) doses of IAV, respectively. Host morbidity (weight loss) (Fig. 4A) and mortality (Fig. 4B) were monitored. Fig. 4C, Quantification of % Ki67⁺ cells in pro-SPC⁺ ATII cells (n=7-8) from *Pex5*^{fl/fl} or *Pex5* ^{Δ Cd11c} mice lung samples after IAV infection at 14 d.p.i.. Fig. 4D, Quantification of PDPN⁺ (staining for alveolar type 1 cells, AT1) area

(left) and pro-SPC⁺ (staining for alveolar type 2 cells, AT2) cell counts (n=4) from *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice lung sections. Fig. 4E, 4F, & 4G, *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice were infected sublethal doses of SARS-CoV-2 MA10 virus. Host morbidity (Fig. 4E) was monitored. Virus titers in the BAL were test (Fig. 4F). Fig. 4G, QuPath quantification of inflamed area in the lungs from 4B (n=4). Fig. 4H&Fig. 4I, *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice were infected with sublethal doses (n=8-9) (Fig. 4H) and lethal (n=15-20) (Fig. 4I) of SARS-CoV-2 MA30 virus, respectively. Host morbidity (4H) and mortality (4I) were monitored. Fig. 4J, Quantification of pro-SPC⁺ AT1 cell counts and % Ki67⁺ cells in pro-SPC⁺ AT2 cells (n=7-9) from *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice lung sections at 7 d.p.i. Data are representative of at least two independent experiments with similar results. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.001.

[0014] FIGURE 5. divided as Figs. 5A-5B illustrates that macrophage peroxisome deficiency in myeloid cells causes increased host morbidity after IAV infection. Fig. 5A, *Pex5^{fl/wt}* (n=6) or *Cd11c^{cre}Pex5^{fl/wt}* (*Pex5* heterozygous, n=7) mice were infected sublethal doses of IAV, and morbidity were monitored. Fig. 5B, *Pex5^{fl/fl}* (n=11) or *Pex5^{ΔCd11c}* (n=12) mice at 4 d.p.i of IAV, the lung immune cell numbers were determined in the BALF.

[0015] FIGURE 6. scRNA-seq analysis of lung cells from infected *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice. Proportions of major cell types from *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice at different timepoints assayed in scRNA-seq data.

[0016] FIGURE 7. divided as Figs. 7A-7D demonstrate that peroxisomes function to maintain AM mitochondria fitness and wound-healing function. Figs. 7A&B, AMs from *Pex5^{fl/fl}* and *Pex5^{ΔLys2}* or *Pex5^{ΔCd11c}* mice, were stimulated with or without Poly(I:C) *in vitro*. Oxygen consumption rate (OCR) of AMs (Fig. 7A). Flow cytometry plots showing mitochondrial mass by MitoTracker Green versus MitoTracker Deep Red in AMs on the left and quantification on the right (Fig. 7B). Fig. 7C, Wound healing rate in peroxisome producing and peroxisome-deficient AMs. Treatment with iMito-ROS in peroxisome-deficient AMs restored their ability to promote wound closure in MLE-12 cells upon AM co-culture. Fig. 7D, Schematic of peroxisome deficient AMs impaired the regeneration of alveolar epithelial. Data are representative of at least two independent experiments with similar results (Fig. 7A-7D). **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.001.

[0017] FIGURE 8. divided as Figs. 8A-8G illustrates that macrophage peroxisome dysfunction leads to the accumulation of dysplastic epithelial progenitor cells and chronic lung sequelae. Fig. 8A, UMAP showing clusters of AT1 and AT2 cells from scRNA-seq analyzed lung cell clusters. Fig. 8B, Proportions of KRT8^{high}-DATP^{s-like} and KRT5-basal progenitor cells

in lung epithelial cells from scRNA-seq data. Fig. 8C, Quantification of KRT8^{high} and KRT5⁺ area from *Pex5^{fl/fl}* and *Pex5^{ΔCd11c}* mice lung samples after IAV infection at day 14. Fig. 8D, Immunoblot of α-SMA and Collagen I of the lungs from IAV-infected *Pex5^{fl/fl}* of *Pex5^{ΔCd11c}* mice (n=6) at 62 d.p.i. Fig. 8E, Immunoblot of α-SMA and Collagen I of the lungs from SARS-CoV-2 MA30 virus infected *Pex5^{fl/fl}* of *Pex5^{ΔCd11c}* mice (n=5-6) at 21 d.p.i. Fig. 8F, Experimental design for 2D culture of primary murine AT2 cells for 3 days with or without indicated conditioned media from AMs stimulated with or without Poly(I:C). Fig. 8G, gene expression of transitional cell markers. Data are representative of at least two independent experiments with similar results. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.001.

[0018] FIGURE 9 divided as Figs. 9A-9F illustrates how pharmacological promotion of peroxisome biogenesis enhances lung repair and limits the development chronic diseases after influenza or SARS-CoV-2 infection. Fig. 9A, Quantification of PMP70 staining density, % PMP70⁺ cells and LC3B staining density in in whole slides (n=4). Fig. 9B, IAV infected C57BL/6 mice (n=10) with or without 4-PBA treatment *in vivo*. Host mortality was monitored. Fig. 9C, After IAV infection, *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice were treated vehicle or 4-PBA. Host mortality was monitored. Fig. 9D, SARS-CoV-2 MA30 virus infected C57BL/6 mice (n=33-38) were treated with vehicle or 4-PBA *in vivo* from day 1 to 6. Host mortality was monitored. Fig. 9E, Schematic of peroxisome deficient AMs impaired the regeneration of alveolar epithelial. Fig. 9F, Number or area of cells in response to 4-PBA treatment. Data are representative of at least two independent experiments with similar results. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.001.

[0019] FIGURE 10. Pharmacological promotion of peroxisome biogenesis in alveolar macrophages. Top, schematic of IFNγ-treated mouse AMs with or without 4-PBA presence. Bottom, immunoblot analysis of indicated peroxisomal proteins in AMs with or without 4-PBA presence.

[0020] FIGURE 11 divided as Figs. 11A-11J demonstrates suppression of inflammation and promotion of tissue repair after the resolution of influenza or SARS-CoV-2 infection by 4-PBA treatment. Host mortality was monitored. (Figs. 11A-11E) IAV infected aged female C57BL/6 mice (n=10-13) were treated with vehicle or 4-PBA *in vivo* from day 14 to 20. (Fig. 11A) Schematic of experimental design. (Figs. 11B and 11C) Quantification of KRT8^{high} areas (Fig. 11B), pro-SPC+ counts, and PDPN+ areas (Fig. 11C) in lung sections at 42 d.p.i. (Fig. 11D) Representative H&E and Masson's Trichrome images of lung sections at 42 d.p.i. (Fig. 11E) Top, immunoblot of α-SMA and Collagen I from the lungs. Bottom, Quantification of α-SMA

and Collagen I expression levels from immunoblots. (Figs. 11F-11J) SARS-CoV-2 MA30 infected middle aged C57BL/6 mice (n=40), 14 survived mice were treated with vehicle (n=6) or 4-PBA (n=6) *in vivo* from day 10 to 16. (Figs. 11G and 11H) Quantification of KRT8high areas (Fig. 11G), pro-SPC+ counts, and PDPN+ areas (Fig. 11H) in lung sections at 21 d.p.i with SARS-CoV-2 MA30. (Fig. 11I) Representative H&E and Masson's Trichrome images of lung sections at 21 d.p.i. (Fig. 11J) Top, immunoblot of α -SMA and Collagen I from lungs in (Fig. 11E). Bottom, Quantification of α -SMA and Collagen I protein expression from immunoblots. Data are representative of at least two independent experiments or pooled from at least two independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

DETAILED DESCRIPTION

TERMINOLOGY

[0021] Prior to describing the disclosure in detail, the following terms may be helpful. Unless otherwise specified all terms carry their ordinary meaning, accepted in the art of pharmaceutical formulations or methods of treating bacterial or viral infections in patients or those exposed to injurious conditions (*e.g.*, smoke inhalation, fine particles, caustic fumes, silicosis, etc.).

[0022] Recitation of ranges of values are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. The endpoints of all ranges are included within the range and independently combinable. All methods described herein can be performed in a suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or language indicating an example (*e.g.*, "such as"), is intended merely for illustration and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0023] The terms "a" and "an" do not denote a limitation of quantity, but rather denote the presence of at least one of the referenced items.

[0024] The term "about" is used synonymously with the term "approximately." As one of ordinary skill in the art would understand, the exact boundary of "about" will depend on the component of the composition. Illustratively, the use of the term "about" indicates that values slightly outside the cited values, *i.e.*, plus or minus 0.1% to 10%, which are also effective and

safe are included in the value. Thus, compositions slightly outside the cited ranges are also encompassed by the scope of the present claims. The term also includes and can encompass those tolerances and fluctuations seen when manufacturing a product according to embodiments disclosed herein.

[0025] The terms “comprising,” “including,” and “containing” are non-limiting. Other non-recited elements may be present in embodiments claimed by these transitional phrases. Where “comprising,” “containing,” or “including” are used as transitional phrases other elements may be included and still form an embodiment within the scope of the claim. The open-ended transitional phrase “comprising” encompasses the intermediate transitional phrase “consisting essentially of” and the close-ended phrase “consisting of.”

[0026] Where elements are presented in list format (*e.g.*, in a Markush group), it is understood that each possible subgroup of the elements is also disclosed, and any one or more elements can be removed from the list or group.

[0027] It is also understood that, unless clearly indicated to the contrary, in any method described or claimed herein that includes more than one act or step, the order of the acts or steps of the method is not necessarily limited to the order in which the acts or steps of the method are recited, but the disclosure encompasses embodiments in which the order is so limited.

[0028] It is further understood that, in general, where an embodiment in the description or the claims is referred to as comprising one or more features, the disclosure also encompasses embodiments that consist of, or consist essentially of, such feature(s).

[0029] It is also understood that any embodiment of the disclosure, *e.g.*, any embodiment found within the prior art, can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification.

[0030] Headings are included herein for reference and to aid in locating certain sections. Headings are not intended to limit the scope of the embodiments and concepts described in the sections under those headings, and those embodiments and concepts may have applicability in other sections throughout the entire disclosure.

[0031] “Providing” means giving, administering, selling, distributing, transferring (for profit or not), manufacturing, compounding, or dispensing.

[0032] “Treatment,” as used herein includes providing a pharmaceutical agent effective to promote peroxisome biogenesis to: (a) reduce probability a disease or a symptom of a disease from occurring in a patient who is be predisposed to the disease but has not yet been diagnosed

as having it (*e.g.* reduce the likelihood or extent of lung, brain or liver injury in a patient suffering from a viral infection; (b) inhibiting the disease, *i.e.* arresting its development; and (c) relieving the disease, *i.e.*, causing regression of the disease. “Treating” and “treatment” also means providing a therapeutically effective amount of a pharmaceutical agent effective to promote peroxisome biogenesis and at least one additional active agent to a patient having or susceptible to lung, brain, or liver injury. For example, where the additional active agent is an anti-inflammatory or antiviral agent.

[0033] Pharmaceutical preparations or compositions described or used herein may further comprise coloring or stabilizing agents, osmotic agents, antibacterial agents, or any other substances if such substances do not interfere with the function of the composition. The pharmaceutical compositions of the instant disclosure, can, for example, be formulated as a solution, suspension, or emulsion in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human albumen. Liposomes may also be used. The vehicle may contain additives that maintain isotonicity (*e.g.*, sodium chloride or mannitol) and chemical stability (*e.g.*, buffers and preservatives). It should be appreciated that endotoxin contamination should be kept at a safe level, for example, less than 0.5ng mg⁻¹ protein. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by the United States Food and Drug Administration Office of Biological Standards. The formulations may be sterilized by commonly used techniques such as filtration.

[0034] The phrase “pharmaceutically acceptable” refers to substances and compositions which do not produce an adverse, allergic, or otherwise untoward reaction when administered to an animal, or a human, as appropriate. A substance which caused or produced any of these adverse effects would be classified as “biologically harmful” within the scope of the present disclosure. Pharmaceutically acceptable substances and compositions include, but are not limited to solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents. Except where incompatible with the disclosure the use of any conventional ingredient is contemplated. Furthermore, supplementary active ingredients which serve some other pharmacologically expedient purpose can also be incorporated into the instant compositions without departing from the broader scope of the instant disclosure.

[0035] The effective dose and method of administration of a particular embodiment of the instant disclosure may vary based on the individual patient and stage of any present diseases (*e.g.*, breast cancer, HIV, respiratory system status, and other co-morbidities), as well as other

factors known to those of skill in the art. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

[0036] Toxicity and safe dosage levels may be determined through the determination of dose-limiting toxicities (DLTs) and the overall DLT-rate (*e.g.*, such as in the context of a clinical trial). In certain embodiments compositions with a DLT rate less than 25% are considered safe.

[0037] Effectiveness of embodiments disclosed herein may further be evaluated through cohort studies and examination of the recurrence-free survival (RFS) rate at chosen time intervals.

[0038] A “therapeutically effective amount” of a pharmaceutical combination of this disclosure means an amount effective, when administered to a patient, to provide a therapeutic benefit such as an amelioration of symptoms, *e.g.*, an amount effective to decrease the symptoms of a lung, brain, or liver injury. If required, embodiments may include one or more pharmaceutically acceptable excipients such as are known in the art.

ABBREVIATIONS

AM	Alveolar Macrophage
BAL	Bronchoalveolar Lavage
BAL	Bronchoalveolar Lavage Fluid
BMDM	Bone Marrow-Derived Macrophages
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
IAV	Influenza A Virus
KRT5	Keratin 5
KRT8	Keratin 5
Mito-ROS	Mitochondrial Reactive Oxygen Species

MLE	Mouse Lung Epithelial cells
PDPN	Podoplanin
SPC	Surfactant protein C
PPAR	Peroxisome Proliferator-Activated Receptor
RIPA	Radioimmunoprecipitation buffer
TCID50	Tissue Culture Infectious Dose 50

[0039] Peroxisomes are metabolic organelles that execute roles in host immune responses that are not widely appreciated. We have observed high levels of peroxisomes in lung macrophages, with their quantity and size markedly altered after severe COVID-19 in both humans and mice. We found that heightened interferon responses diminished peroxisome biogenesis and enhanced pexophagy, resulting in substantial remodeling of the macrophage peroxisome compartment following severe viral infection. Notably, selective ablation of peroxisomes in macrophages increased acute morbidity and mortality during influenza and SARS-CoV-2 infections. This deficiency in macrophage peroxisomes did not significantly alter acute inflammatory responses or viral clearance but compromised the resolution of inflammation and impaired regeneration of the alveolar epithelium.

[0040] Peroxisomes are essential for degrading long-chain fatty acids, enhancing mitochondrial health, and supporting macrophage repair mechanisms. Moreover, peroxisome activity in macrophages is instrumental in preventing inflammasome activation and IL-1 β release, thereby facilitating the proper differentiation of transitional epithelial progenitors into functional alveolar epithelial cells after injury. Consequently, dysfunction of peroxisomes in macrophages contribute to chronic tissue pathology following acute viral infections including influenza and SARS-CoV-2. Strikingly, we also observed a negative correlation between macrophage peroxisome levels and the long-term persistence of dysplastic progenitor cells in the lungs of COVID-19 patients with fibrotic sequelae. We provide methods of pharmacologically enhancing peroxisome biogenesis and thereby augmenting lung regeneration, dampening acute diseases and alleviating chronic sequelae post-viral infection. This disclosure provides a methods of treating lung damage due to respiratory viral infections and a peroxisome-centric therapeutic strategy to manage both acute and long-term consequences of COVID-19.

[0041] *Peroxisome compartment remodeling in macrophages following severe acute COVID-19*

To explore the potential roles of peroxisomes in host immune responses, we first utilized

publicly available single-cell RNA sequencing (scRNA-seq) datasets to map the peroxisome pathway in the lung and found that alveolar macrophages (AMs) expressed high levels of peroxisome pathway genes compared with other lung cell types in different species. Moreover, lung macrophages appeared to have the greatest expression levels of peroxisome-associated genes compared with other tissue macrophages in both human and mice. Notably, the expression of peroxisome-associated genes was diminished in both total lung macrophages and the AM compartment in severe, but not moderate, COVID-19. Consistent with this, peroxisomal proteins, PEX5, PMP70 and PEX14, were significantly decreased in the lung cells isolated from acute COVID-19 autopsy samples compared with non-COVID-19 controls (Figs. 1A-1B). Similarly, viral infection in mice also caused diminished PEX5, PMP70 and PEX14 expression in alveolar macrophages (Figs. 1C-1D).

[0042] To further examine whether the peroxisome compartment is altered in macrophages after severe COVID-19, we performed immunofluorescence (IF) on PEX14 and CD68 or CD169 in non-COVID-19 control lungs and acute COVID-19 autopsy lungs. Decreased peroxisome containing total CD68⁺ macrophages and resident AMs (CD169⁺) were observed in COVID-19 autopsy lungs (Figs. 2A-2B). Furthermore, the peroxisome numbers in those macrophages that still contain peroxisomes were decreased in COVID-19 lungs compared to those of control lungs. At the single cell level, we observed that macrophages from COVID-19 lungs had bigger peroxisome aggregates, while most peroxisomes in control lung macrophages were smaller punctate, which were generally uniform in size and dispersed throughout the cytoplasm (Figs. 2A-2B). These data indicate that severe COVID-19 causes extensive remodeling of the peroxisome compartment in lung macrophages, as evidenced by either the loss of cellular peroxisomes or decreased numbers of peroxisomes per cells that is accompanied with increased peroxisome size or aggregates.

[0043] We next sought to examine whether peroxisome alterations are observed during SARS-CoV-2 infection in animal models. To this end, we infected wild-type (WT) male mice (Male mice are more susceptible to SARS-CoV-2 infection) with increasing doses of SARS-CoV-2 MA30 virus to mimic mild, severe, or lethal infections. We then assessed the alteration of the peroxisome compartment in AMs. By day 4 post-infection (d.p.i), we noticed peroxisome compartment remodeling in mouse AMs, similar to what we observed in human cases. Most peroxisomes in naïve AMs were punctate in shape, generally uniform in size, and dispersed throughout the cytoplasm. There were no changes observed in the peroxisomes of AMs from the mild infection group. In case of severe infection, the total levels of peroxisomes in AMs

were decreased compared with those of naïve and mild-infected mice. Nevertheless, heterogeneous peroxisome present in AMs following SARS-CoV-2 infection. While peroxisomes in some AMs resembled that of naïve AMs, others displayed reduced levels, with enlarged or aggregated peroxisomes (Fig. 2C). Strikingly, a lethal SARS-CoV-2 infection resulted in a pronounced decrease in peroxisome-containing AMs and much larger peroxisome aggregates in those AMs still containing peroxisomes.

[0044] To understand whether other important respiratory viral pathogens can also cause extensive remodeling of the peroxisome compartment in AMs, we infected WT mice with varying doses of influenza virus A/PR8/34 (IAV) and collected AMs at 4 or 8 d.p.i. There were no detected differences in the AM peroxisome compartment between naïve and mild infection groups, while severe infection caused heterogeneous peroxisome remodeling in AMs (Fig. 2D). Similar to what was observed in lethal SARS-CoV-2 infection, lethal IAV infection caused a drastic decrease in peroxisome containing AMs, and increased peroxisome size or aggregates in those AMs that still contained peroxisomes (Fig. 2D). The reduction in peroxisome protein expression after lethal IAV infection was further confirmed using western blot (Figs. 1C-1D). Taken together, our results reveal that respiratory viral infections induce marked changes in the peroxisome compartment of lung macrophages in both humans and mice, and with extent of alterations modulated by the severity of infection.

[0045] *Interferons inhibit peroxisome biogenesis and promote pexophagy in lung macrophages.* To investigate the mechanisms through which viral infections drive peroxisome remodeling in lung macrophages, we exposed mouse AMs *in vitro* to stimuli that mimic the various signals triggered by respiratory viral infections in the host. Type I and type II interferons (IFNs) (IFN α or IFN γ), but not type III IFNs or other stimuli, decreased expression levels of peroxisomal proteins (Fig. 3A). Furthermore, loss of peroxisomes, decreased numbers of peroxisomes accompanying increased peroxisome size or aggregates were observed in IFN α or IFN γ treated AMs. Notably, IFN α or IFN γ treatment of AMs significantly inhibited peroxisomal gene expression (Fig. 3B), with IFN γ inducing more pronounced changes. To test whether IFN α or IFN γ drives peroxisome remodeling during severe respiratory viral infection *in vivo*, we blocked IFN α or IFN γ signaling through the administration of neutralizing anti-IFN γ or anti-IFN α/β receptor blocking antibodies (Abs) after lethal IAV infection. IFN γ neutralization resulted in increased peroxisome PMP70 levels, reduced percentage of peroxisome-deficient AMs, and decreased levels of peroxisome aggregates in AMs compared with those of AMs from the control group. IFN α receptor blockade, while effective, exerted

less profound effects compared to IFN γ neutralization. These results indicate that exuberant IFN responses, particularly type II IFN responses, cause peroxisome remodeling in lung macrophages after respiratory viral infection.

[0046] To confirm the role of IFN γ in remodeling the peroxisome compartment in human AMs, we treated human AMs with IFN γ *in vitro* and observed a pattern similar to murine AMs after IFN γ treatment. IFN γ caused either complete loss or a reduction in the numbers of peroxisomes accompanying an increase in peroxisome size or aggregates. After IFN γ treatment, there was a decrease or loss in peroxisomes, which may not solely be explained by the inhibition of peroxisome biogenesis (Fig. 3B). Furthermore, the accumulation of peroxisome aggregates indicates potential induction of pexophagy, a selective autophagy targeting peroxisomes. In support of the idea, we noted IFN γ treatment led to increased PMP70 ubiquitination, which was known to trigger pexophagy. Additionally, elevated expression of the autophagosome marker, LC3B, was observed which appeared to colocalize with PMP70 in both human and mouse AMs after IFN γ treatment. Taken together, these findings indicate that IFNs orchestrate peroxisome remodeling by inhibiting peroxisome biogenesis and facilitating peroxisome degradation (Fig. 3C).

[0047] *Macrophage peroxisomes are required for alveolar repair post viral injury.* We next employed *Cd11c^{cre}Pex5^{flox/flox} (Pex5 Δ Cd11c)* mice and *Lyz2^{cre}Pex5^{flox/flox} (Pex5 Δ Lyz2)* mice to study the phenotypic consequences of peroxisomal absence in lung macrophages as *Pex5* is essential for peroxisome biogenesis. Observations in naive mice indicated that *Pex5*-deficient AMs exhibited a slight decrease in Siglec F but increased CD11c expression. *Pex5* deficiency did not substantially affect cell numbers of AM or other myeloid cell populations, with no observed effect on AM proliferation, phagocytosis or lung histology under homeostasis. We next proceeded to assess the pathophysiological functions of peroxisomes in lung macrophages after viral infection. Upon exposure to sublethal or lethal IAV infection doses, *Pex5 Δ Cd11c* mice exhibited increased morbidity and reduced survival (Figs. 4A, 4B). These effects were absent in *Pex5^{flox/wt}* mice or heterozygous (*Cd11c^{cre}Pex5^{flox/wt}*) mice (Fig. 5A). The increased vulnerability of *Pex5 Δ Cd11c* mice to infection was independent of viral load and clearance, suggesting that lung macrophage peroxisome deficiency does not impair host antiviral immunity *in vivo*. Examination of the BAL (bronchoalveolar lavage) and lungs at intervals of 4, 8, and 14 d.p.i revealed a marked increase in the infiltration of inflammatory monocytes (Ly6C^{high}) and neutrophils. Yet, no discernible effect was observed on the counts of dendritic cells (DCs), CD4⁺ T cells, total and virus-specific CD8⁺ T cells. Same escalation of

vulnerability to IAV infection in *Pex5^{ΔLyz2}* mice was observed (Fig. 5B). Additionally, there were no differences were seen in DCs, CD4⁺ T cells, and CD8⁺ T cell populations in lung-draining lymph node (dLN) or spleen at 4 d.p.i in *Pex5^{ΔCd11c}* mice. Together, these data indicate that *Pex5* deficiency in CD11c⁺ cells does not affect the induction of antiviral adaptive T cells. Notably, evaluation of BAL cytokine and chemokine levels revealed similar levels in *Pex5^{ΔCd11c}* and control mice at 4 and 8 d.p.i, but a marked increase in proinflammatory mediators at 14 d.p.i in *Pex5^{ΔCd11c}* mice. These data suggested that macrophage *Pex5* deficiency did not drastically affect acute inflammatory responses but impaired the resolution of inflammation after viral clearance. Consistently, *Pex5^{ΔCd11c}* mice had increased inflammatory lesions in the lungs at 14, but not 4 or 8 d.p.i compared to *Pex5^{flx/flx}* mice. Additionally, there was an increase in red blood cells (RBCs) and total protein in the BAL of IAV-infected *Pex5^{ΔCd11c}* mice at 14 d.p.i, indicating enhanced pulmonary vascular leakage in these mice. Together, these data suggest that macrophage peroxisomes play a critical role in modulating the resolution of pulmonary inflammation and facilitating tissue recovery following viral injury.

[0048] To delve into the mechanisms of how macrophage peroxisomes confer protection in the lung, we performed single-cell RNA sequencing (scRNA-seq) analyses on lungs from both naive (uninfected, day 0) and IAV-infected *Pex5^{flx/flx}* and *Pex5^{ΔCd11c}* mice, specifically 8 and 14 d.p.i. Consistent with our previous data, scRNA-seq analyses did not reveal any stark differences in the cellular and molecular profiles of naive *Pex5^{flx/flx}* and *Pex5^{ΔCd11c}* mouse lungs during homeostasis. However, corroborating the increased prevalence of lung damage, we noticed a heightened proportion of inflammatory monocytes (Ly6C^{high}), neutrophils, and monocyte-derived macrophages (MDM) present in the lungs of infected *Pex5^{ΔCd11c}* mice compared to those of infected control mice. Additionally, there was a noticeable decrease in alveolar type 2 (AT2) cells and AMs post-infection in *Pex5^{ΔCd11c}* mice, with these effects being more pronounced at 14 days post-infection (Fig. 6). In line with previous observations, the lungs of *Pex5^{ΔCd11c}* mice displayed an enrichment of inflammation-associated gene sets (such as IFN γ and IFN α responses, TNF α , IL-6_JAK_STAT3 and IL2_STAT5 signaling pathways) and cellular stress profiles (such as hypoxia, p53, reactive oxygen species, UV response and apoptosis), with a concomitant down-regulation of cell proliferation pathways (E2F and MYC targets, and G2M checkpoint).

[0049] Within the AM compartment, *Pex5^{flx/flx}* and *Pex5^{ΔCd11c}* AMs display minimal gene differences in the absence of infection. However, a substantial divergence in gene expression

was observed after viral infection. Through functional annotation, a high enrichment of innate immune response, inflammation-associated gene sets, and cellular stress profiles was evident in *Pex5^{ΔCd11c}* AMs. This is further supported by *in vitro* bulk RNA-seq data, which aligns with scRNA-seq findings, demonstrating an amplification of inflammation-associated pathways and innate immune reactions in *Pex5*-deficient AMs, irrespective of Poly(I:C) treatment. Contrary to a previous study that posited peroxisomal MAVS promoted IFN-independent anti-viral ISG gene expression in mouse embryonic fibroblasts (MEFs), our data from both *in vivo* AMs scRNA-seq and *in vitro* bulk RNA-seq indicate an upregulation of these ISG genes in peroxisome-deficient AMs, suggesting potential cell-type specific anti-viral functions of peroxisomes.

[0050] Noticeably, *Pex5*-deficient AMs exhibited a marked suppression of pathways tied to the regulation of epithelial cell growth, differentiation, proliferation, and wound healing mechanisms. Attenuated tissue development-regulated pathways was also observed in bulk RNA-seq of *Pex5*-deficient AMs with or without Poly(I:C) treatment *in vitro*. We hypothesized that diminished AM wound-healing gene expression may lead to impaired alveolar epithelial repair. In support of that hypothesis, scRNA-seq analysis showed that AT2 cells from *Pex5^{ΔCd11c}* mice had impaired expression of genes associated with proliferation and differentiation, but increased expression of genes associated with inflammation, cellular stress, and cell death. Furthermore, the proportion of Ki67-expressing AT2 cells were decreased in *Pex5^{ΔCd11c}* mice. Utilizing CellChat analysis, we discerned a marked disruption in the molecular interaction map between AMs and AT2 cells after AM peroxisome deficiency following infection. Taken together, these data suggest that macrophage peroxisomes may mediate crucial crosstalk between macrophages and the epithelium to facilitate successful alveolar regeneration.

[0051] Consistent with the idea, IF analysis revealed diminished AT2 cell proliferation (as reflected by % Ki67⁺ among pro-SPC⁺ cells) in *Pex5^{ΔCd11c}* mice at both 8 and 14 d.p.i (Fig. 4C). Furthermore, decreased counts of AT2 and AT1 cells were observed at 14 but not 8 d.p.i (Fig. 4D). These data indicate that macrophage peroxisomes do not affect the peak of acute alveolar damage (*i.e.*, 8 d.p.i), but play a pivotal role in aiding alveolar epithelial repair following lung injury after virus clearance (*i.e.*, 14 d.p.i). To further elucidate the impact of peroxisome deficiency in the wound healing process of alveolar epithelium, we conducted an *in vitro* wound healing assay, assessing the migratory capabilities of MLE-12 alveolar epithelial cells. Remarkably, we observed a significant elevation in the scratch wound

confluence after co-culture with *Pex5^{flax/flax}* AMs relative to the no-AM controls or *Pex5^{ΔCd11c}* AMs, regardless of stimulation with Poly(I:C). Furthermore, 3D organoid co-cultures in which isolated primary AT2 cells were cultured together with either Poly(I:C)-stimulated *Pex5^{flax/flax}* or *Pex5^{ΔCd11c}* AMs revealed increased colony forming efficiency when AT2 cells were co-cultured with WT (*Pex5^{flax/flax}*) AMs, but not with *Pex5^{ΔCd11c}* AMs, compared to control cultures without AMs. Taken together, these data reveal that AM peroxisomes are critical for alveolar epithelium wound healing and regeneration after viral injury.

[0052] Critically, we observed similar increased host morbidity and mortality after SARS-CoV-2 infection (both MA10 and MA30 strains) in lung macrophage peroxisome deficient mice. In *Pex5^{ΔCd11c}* mice, there was a notable increase in weight loss (Fig. 4E, 4H), mortality (Fig. 4I), and lung damage, coupled with elevated immune cell infiltration (Fig. 4G), independent of the viral loads (Fig. 4F). Notably, a decrease in the proliferation and counts of AT2 cells was observed in *Pex5^{ΔCd11c}* mice post SARS-CoV-2 infection (Fig. 4J). These findings together highlight the pivotal role of AM peroxisomes in mediating alveolar tissue repair after severe influenza and SARS-CoV-2 infections.

[0053] *Peroxisomes are required to maintain AM mitochondrial fitness and a wound-healing gene program.* Peroxisomes are critical to lipid metabolism as they are responsible for ether lipid synthesis and the breakdown of very long chain fatty acids (VLCFAs) before these lipids can be further metabolized in mitochondria. Dysregulation in peroxisome function can potentially disrupt lipid balances, resulting in significant cellular stress and anomalies. To discern whether peroxisome deficiency alters lipid and/or other metabolite profiles in AMs, we measured cellular metabolome profiles of *Pex5^{flax/flax}* and *Pex5^{ΔLyz2}* AMs, with or without Poly(I:C) stimulation *in vitro*. Consistent with their roles in lipid metabolism, peroxisome deficiency led to aberrant accumulation of VLCFAs and a decline in ether lipid production. This pattern was also noted in *Pex5^{ΔLyz2}* bone marrow-derived macrophages (BMDMs) when compared to *Pex5^{flax/flax}* BMDMs, albeit to a lesser extent. In line with these findings, lipid ontology analysis revealed a notable decrease in ether-bond lipids in *Pex5^{ΔLyz2}* AMs, a trend not seen in *Pex5^{ΔLyz2}* BMDMs. These data indicate that peroxisomes perform differential lipid metabolic functions in distinct macrophage subsets.

[0054] Given that products degraded from VLCFA can serve as substrates for mitochondrial metabolism and ether lipids, particularly plasmalogens, are crucial components of organelle (including mitochondrion) membranes, we reasoned that peroxisome impairment may alter mitochondrial metabolism and fitness. Consistent with this notion, bulk RNA-seq analysis

revealed that peroxisome deficiency resulted in suppressed mitochondria-related functional pathways. Upon assessing the mitochondrial oxygen consumption rate (OCR), no discernable differences emerged between *ex vivo* $Pex5^{flav/flav}$ and $Pex5^{Al_{yz}2}$ AMs during homeostasis. However, post *in vitro* culture, with or without Poly(I:C) stimulation, $Pex5^{Al_{yz}2}$ AMs exhibited reduced mitochondrial respiration capability and a decline in extracellular acidification rate (ECAR) (Fig. 7A). These data suggest that peroxisomes are required for mitochondrial metabolic function under stress. While we also detected a decrease in ECAR in $Pex5^{Al_{yz}2}$ BMDMs, the OCR remained consistent between $Pex5^{flav/flav}$ and $Pex5^{Al_{yz}2}$ BMDMs. Relative to AMs, both BMDMs and peritoneal macrophages (PMs) contain fewer peroxisome proteins, potentially explaining the tissue-specific function of peroxisomes among macrophage populations. Consistent with the impaired mitochondrial respiration observed in peroxisome-deficient AMs, there was an increase in depolarized mitochondria and a decrease in healthy mitochondrion numbers (Fig. 7B). Aligning with the observations, peroxisome impairment led to enhanced mitochondrial reactive oxygen species (ROS) levels, accompanied with an increase in total intracellular ROS levels.

[0055] Maintaining proper mitochondrial fitness and function is paramount for facilitating a wound healing gene program in macrophages. We therefore examined whether peroxisome deficiency could result in diminished pro-repair gene expression by macrophages. To this end, genes associated with aiding epithelial regeneration and wound healing were found to be markedly downregulated in peroxisome-deficient AMs, consistent with the data that peroxisome-deficient AMs displayed reduced wound confluence and diminished proliferation of alveolar epithelial cells during injury (*see*, Figs. 4 and 5). Notably, treatment with a mitochondrial ROS inhibitor (iMito-ROS) appeared to reverse these deficits in peroxisome-deficient AMs, rescuing the expression of wound healing genes. Furthermore, treatment of iMito-ROS in peroxisome-deficient AMs restored their ability to promote wound closure in MLE-12 cells upon AM co-culture (Fig. 7C). These findings indicate that peroxisome deficiency hinders lung macrophage wound healing and pro-repair functions after severe viral infection, at least in part via the disruption of lipid metabolism and mitochondrial health (Fig. 7D).

[0056] *Macrophage peroxisome impairment leads to the accumulation of dysplastic transitional progenitor cells and chronic lung sequelae.* Given the reduction of AT2 and AT1 cells in $Pex5^{ACd11c}$ mice following viral infection-mediated alveolar injury, we hypothesized that peroxisome dysfunction in AMs may disrupt AT2- to-AT1 trans-differentiation. Within

alveolar epithelial cells, we identified six subsets of alveolar epithelial cells and a contaminant fibroblast population in the scRNA-seq dataset (Fig. 8A). Pseudotime analysis supported the transition of AT2 into AT1 cells via primed AT2 cells and KRT8^{high} damage-associated transient progenitors (DATPs) (Fig. 8A), corroborating previous studies. Importantly, viral infection in *Pex5^{ΔCd11c}* mice induced an increase in proportions of both KRT8^{high}-DATPs and KRT5⁺ basal cells (Fig. 8B). This increase was validated using immunostaining of lung sections at 14 d.p.i (Fig. 8C). Strikingly, the sustained presence of KRT8^{high} transitional cells and ectopic KRT5⁺ basal cells was observed in the *Pex5^{ΔCd11c}* mice at both 35 and 62 d.p.i with IAV, suggesting that peroxisome deficiency resulted in prolonged presence of dysplastic epithelial progenitors after severe viral injury. Concurrently, there were decreased counts of both AT1 and AT2 cells at these time points. In addition to the observed cellular changes, extensive lung tissue inflammation and collagen deposition were noted in *Pex5^{ΔCd11c}* mice. Increased neutrophilia was observed, further supporting continuous inflammatory cell infiltration in *Pex5^{ΔCd11c}* mice. Additionally, immunoblot revealed increased collagen I and α -SMA (smooth muscle actin) presence in the lungs of *Pex5^{ΔCd11c}* mice, confirming enhanced lung fibrosis in these mice (Fig. 8D). Furthermore, SARS-CoV-2 MA30 infection in *Pex5^{ΔCd11c}* mice led to a rise in KRT8^{high}-DATPs, aggravated lung fibrosis, and heightened inflammatory infiltration, alongside diminished AT1 and AT2 cells at 21 d.p.i compared to Cre⁻ littermate controls (Fig. 8E). Collectively, these data reveal that macrophage peroxisome impairment leads to the prolonged accumulation of dysplastic epithelial progenitor cells and sustained lung pathology after acute viral infection.

[0057] We next employed a 2D primary AT2 cell culture model known to induce spontaneous differentiation into AT1 cells through the transitional cell stage. To this end, primary AT2 cells were incubated with conditioned media from cultured *Pex5^{lox/flox}* and *Pex5^{ΔCd11c}* AMs with or without Poly(I:C) stimulation. We observed reduced AT1 cell marker expression (including *Vegfa*, *Aqp5* and *Clic5*), but a significant increase in the expression of transitional cell markers (such as *Hif1a*, *Cldn4*, *Slc16a3* and *Slc2a1*)¹⁶ in cultured AT2 cells with conditioned media from peroxisome-deficient AMs, particularly after Poly(I:C) stimulation (Fig. 8F & Fig. 8G). These data indicate that peroxisome deficient macrophages directly promote transitional cell development and arrest their further differentiation into AT1 cells. We next sought to examine the potential molecular mechanisms by which macrophage peroxisome deficiency promote transitional cell accumulation. To this end, IL-1 β has been shown to stimulate the expansion of transitional KRT8^{high} cells. IL-1 β levels were significantly

increased in BAL from *Pex5^{ΔCd11c}* mice after virus infection. Furthermore, we observed enhanced IL-1 β release by cultured *Pex5*-deficient AMs compared with WT AMs. Consistent with these observations, increased Caspase-1 activity and cell death were observed in *Pex5*-deficient AMs compared with WT AMs *in vitro* and *in vivo*, suggesting that peroxisome deficiency resulted in increased inflammasome activation and pyroptosis. Of note, peroxisome deficiency in BMDMs had minimal impact on Caspase-1 activity and cell death. We next asked whether increasing IL-1 β release by peroxisome-deficient AMs contributed to the accumulation of the transitional cell state. To assess this, we neutralized IL-1 β activity *in vitro*, and found that α IL-1 β treatment diminished the expression of transitional cell markers but increased AT1 marker expression (Fig. 8F, Fig. 8G). These data suggest that peroxisomes guide the proper differentiation of alveolar transitional cells, at least partially through their inhibition of inflammasome activation and IL-1 β release in lung macrophages.

[0058] In our attempt to understand the relevance of these findings to human PASC, we examined macrophage peroxisome levels in lung sections from a cohort of COVID-19 convalescents with persistent pulmonary fibrosis (PASC-PF) that required lung transplantation. We observed pronounced lung tissue inflammation, collagen deposition, and reduced AT1 and AT2 cell levels compared to controls. Moreover, we identified a decline in peroxisome-containing macrophages but observed an increase in KRT8^{high} transitional cells in PASC-PF lungs. A notable negative spatial correlation between peroxisome-containing lung macrophages and KRT8^{high} areas was observed, further indicating a link between peroxisome dysfunction in lung macrophages and dysplastic repair in PASC-PF patients.

[0059] *Pharmacological promotion of peroxisome biogenesis enhances lung repair and limits chronic disease post-acute viral infection.* Our data thus far strongly suggest that macrophage peroxisome deficiency results in impaired resolution of pulmonary inflammation, diminished alveolar regeneration, and the emergence of chronic lung sequelae. As severe viral infection impairs macrophage peroxisome biogenesis and promotes their degradation, we next sought to examine whether the promotion of peroxisome biogenesis could ameliorate virus-induced lung injury and chronic lung disease following respiratory viral infection. To investigate this, we employed a pharmacological approach by using sodium 4-phenylbutyrate (4-PBA), a known agent capable of enhancing peroxisomal biogenesis. *In vitro* treatment of AMs with 4-PBA amplified the expression of peroxisome proteins (Fig. 10). Notably, both human and mouse AMs subjected to 4-PBA treatment demonstrated a substantial reversal of the IFN γ -induced remodeling of the peroxisome compartment. Following treatment, we

observed an increase in overall peroxisome levels, peroxisome containing cells, and the inhibition of pexophagy evidenced by reduced co-localization of PMP70 with LC3B (Fig. 9A). Further extending these observations, 4-PBA treatment *in vivo* (4 - 8 d.p.i.) restored peroxisome biogenesis and remodeling within AMs after IAV infection. Remarkably, mice received 4-PBA exhibited reduced mortality at 14 days after IAV infection (Fig. 9B). However, such benefits were absent in *Pex5^{ΔCdl1c}* mice (Fig. 9C), suggesting that the survival-enhancing effects of 4-PBA post viral infection are contingent on its ability to modulate the peroxisome compartment. Further, 4-PBA treatment regimen from 1 to 6 d.p.i. led to a decline in host mortality following a lethal dose of SARS-CoV-2 MA30 infection (Fig. 9D). Moreover, early interventions using 4-PBA post IAV or SARS-CoV-2 MA30 infections in WT mice markedly alleviated fibrotic responses and dampened immune cell infiltration in comparison to controls at 45 d.p.i or 21 d.p.i, respectively (Fig. 9E) (21 d.p.i was chosen in SARS-CoV-2 model as the virus induced relatively transient sequelae post-acute infection in mice. Overall, our findings underscore the potential therapeutic benefits of promoting peroxisome biogenesis using pharmacological agents to bolster alveolar regeneration and suppress pathological sequelae observed following severe respiratory viral infections.

[0060] To determine the therapeutic potential of 4-PBA in treating chronic tissue sequelae post-viral clearance, we utilized IAV infection in aged WT C57BL/6 mice, which exhibited persistent inflammation and tissue pathology post acute infection. Administering 4-PBA from day 14-day 20 post infection reduced KRT8^{high} cells but increased AT1 and AT2 cells at 42 d.p.i. (Fig. 11, 11A-11C), indicating that 4-PBA treatment post viral clearance restricts the accumulation of dysplastic epithelial progenitors and promotes lung functional regeneration. Consistently, 4-PBA treatment mitigated lung inflammation and tissue collagen deposition (Fig. 11, 11D and 11E). We next utilized SARS-CoV-2 MA30 infection in the middle-aged male mouse model to determine whether the promotion of peroxisome biogenesis by 4-PBA could dampen PASC lung conditions therapeutically. To this end, 4-PBA was administered from 10 to 16 d.p.i at the time after SARS-CoV-2 viral clearance. Remarkably, 4-PBA treatment resulted in diminished KRT8^{high} transitional cell accumulation, improved AT1 and AT2 cell presence (Fig. 11, 11F-11H) and a concurrent reduction in tissue inflammation and fibrosis (Fig. 11, 11I-11J), indicating that 4-PBA promotes lung recovery and dampens PASC lung conditions. Overall, the findings uncover the potential therapeutic benefits of promoting peroxisome biogenesis using pharmacological agents to augment alveolar regeneration and limit chronic sequelae after severe respiratory viral infections including PASC.

[0061] Discussion. The mechanisms “driving” the development of post-infection sequelae remain largely enigmatic due to the relative lack of comparative studies on human samples and proper animal models. Our studies have pinpointed a crucial defect within the macrophage peroxisome compartment as a key driver of chronic lung sequelae, through careful analyses of human PASC samples and relevant animal models. We further discovered that compromised peroxisome function in macrophages disrupts proper alveolar regeneration and leads to the accumulation of dysplastic epithelial progenitor cells, culminating in persistent pulmonary inflammation and fibrosis.

[0062] Alveolar damage resulting from respiratory infections prompts a variety of repair mechanisms to restore the affected epithelium. Post-viral infection lung injuries cause a proportion of the AT2 cells to proliferate and differentiate into AT1 cells, a process intricately regulated by signals within the alveolar niche. AMs within this niche are crucial in resolving inflammation, supporting AT2 cell renewal, and facilitating repair by secreting a cocktail of pro-healing factors. Our research underscores the importance of peroxisomes in regulating these pro-recovery functions of AMs, particularly via the modulation of genes involved in wound healing. While our findings illuminate the role of peroxisome function in upregulating genes tied to AT2 cell renewal, whether these interactions are mediated by cytokines, metabolite signaling through peroxisomes, or direct AM-AT2 cell interactions remains unknown.

[0063] Beyond influencing AT2 cell renewal, our data also reveal that proper peroxisome function prevents the accumulation of KRT8^{high} transitional cells in the lung. Normally, these cells are a physiological feature of AT2 cell trans-differentiation following injury, however, aberrant accumulation and persistence is indicative of dysplastic lung remodeling and fibrosis. Our findings suggest that impaired peroxisomes in macrophages could either promote AT2 differentiation into transitional cells or impede their progression into AT1 cells. We have recently identified a disrupted immune-epithelial progenitor niche in the lungs of PASC patients and an animal model of post-viral sequelae, characterized by an abnormal accumulation of CD8⁺ T cells and persistent IFN signaling. This environment may perpetuate dysplastic repair by triggering chronic IL-1 β release from lung macrophages. Notably, interferons, especially IFN γ , suppress peroxisome biogenesis and enhance pexophagy, suggesting that T cell-derived cytokines alter organelle function of lung macrophages following viral infection. These findings hint at a peroxisome-centric axis mediating the complex interplay among T cells, macrophages and epithelial progenitors, which drives tissue

sequelae post-acute lung injury. The exact molecular link, however, between IFN γ signaling and peroxisome protein ubiquitination and pexophagy, remains to be clarified.

[0064] The dynamic interaction between peroxisomes and mitochondria is pivotal for cellular metabolism, coordinating the exchange of metabolites and signals. Previous studies have documented mitochondrial dysfunction in various cell types following severe influenza and SARS-CoV-2 infections, including macrophages and epithelial cells, linking this dysfunction to oxidative stress, inflammasome activation, cell death, and immunopathology. These disturbances are central to the pathogenesis of both acute and chronic sequelae of viral infections. Given that peroxisome dysfunction can cause significant mitochondrial stress and dysfunction in AMs, it is tempting to speculate that peroxisome impairment may precede mitochondrial dysfunction in such scenarios. Consequently, targeting peroxisome dysfunction could represent a promising therapeutic strategy for tackling the root cause of impaired lung recovery and persistent tissue sequelae following infection, a notion supported by the efficacy of 4-PBA treatment in mitigating acute and chronic disease after respiratory viral infections. Notably, peroxisomes appear to display a cell type-specific function in different macrophage populations. AMs at homeostasis exhibit higher peroxisome content compared to BMDMs. While BMDMs show metabolite changes in response to peroxisome deficiency, their mitochondrial fitness and survival under stress remain largely unaltered. This difference likely stems from the unique positioning of AMs in the alveoli. In the alveolar space, AT2 cells secrete surfactants, which are catabolized by AMs to maintain lung homeostasis. The higher peroxisome content observed in AMs may be required for proper degradation of lipid-rich surfactant *in vivo*, particularly under stress. Previous studies have also noted the potential for monocyte-derived macrophages to replenish the resident AM pool post-infection, depending on the nature of injury and loss of AMs. This raises a question about whether recruited macrophages can assume AM-like peroxisomal functions upon lung adaptation, and whether pharmacological enhancement of peroxisome biogenesis could facilitate this transition, thereby aiding lung recovery.

[0065] Patients with peroxisome biogenesis disorders, such as Zellweger syndrome, often experience recurrent respiratory issues, leading to high morbidity. Infants with these conditions typically succumb within the first year of life, primarily due to respiratory distress or severe epilepsy. Therefore, it is advised for these patients to receive vaccinations against respiratory viruses. These clinical insights underscore the crucial role of peroxisomes in lung disease progression following viral infections. Although it is difficult to assess the acute and long-term

effects of COVID-19 due to the rarity and early mortality of peroxisome disorders, our research highlights the need for heightened vigilance among patients with these disorders against respiratory viral infections. Furthermore, our findings support a treatment approach centered on peroxisome function to aid in lung recovery post-viral infection. While 4-PBA may affect other pathways or cell types, its efficacy appears largely dependent on peroxisome function, as shown by the unchanged survival rate in treated *Pex5^{-ΔCd11c}* mice.

[0066] We have discovered a critical peroxisome-related mechanism underlying severe acute disease and chronic host sequelae induced by SARS-CoV-2, influenza and possibly other respiratory viruses. We provide a method of treating lung injury, including Covid related lung injury and suppressing long COVID by boosting peroxisome biogenesis and peroxisome function. Moreover, our findings propose that boosting peroxisome biogenesis or function provides a novel therapeutic avenue to enhance lung repair and mitigate chronic conditions following severe respiratory infections.

EXAMPLES

Example 1

[0067] In an envisioned embodiment, a patient experiencing a respiratory viral infection, or who has cleared a respiratory viral infection but is still experiencing symptoms of respiratory system damage is administered a therapeutically effective amount of a pharmaceutical agent that enhances peroxisome biogenesis. In certain instances the patient may be administered an additional antiviral medication and/or an additional anti-inflammatory medication. In certain embodiments, the antiviral medications may be paxlovid or molnupiravir. In certain other embodiments, the anti-inflammatory may be dexamethasone or tocilizumab.

Example 2

[0068] In another envisioned embodiment, a patient may have suffered respiratory system injury due to the exposure of one or more caustic substances. In some instance, the substances may be tobacco smoke, asbestos, or fine particulate matter (*e.g.*, silicosis). In such instances the patients may be administered a therapeutically effective amount of a pharmaceutical agent that enhances peroxisome biogenesis. Additional therapeutic agents may be co-administered or administered as part of an overall treatment plan.

Example 3

[0069] In another envisioned embodiment, a patient may be experiencing a peroxisome biogenesis disorder. An example biogenesis disorder is Zellweger syndrome.

[0070] For any of the embodiments and Examples disclosed herein compatible pharmaceutical agents can include: 4-phenylbutyrate, a monounsaturated fatty acid, such as an omega-3 fatty acid (e.g. DHA or EPA) or an omega-6 fatty acid, or an alpha, beta, or gamma PPAR (peroxisome proliferator-activated receptor) agonist. In still other additional embodiments, the PPAR agonists can include: clofibrate, gemfibrozil, ciprofibrate, bezafibrate, fenofibrate, pioglitazone, rosiglitazone, or lobeglitazone.

GENERAL METHODS

[0071] *Ethics and biosafety.* This study received full approval from the Institutional Review Board Committee at Cedars-Sinai Medical Center and the University of Virginia. All animal experiments were conducted in the animal housing facilities of the University of Virginia (UVA; Charlottesville, VA). Unless otherwise noted, experiments utilized adult mice that were matched for sex and age. The UVA Institutional Animal Care and Use Committee granted approval for all animal experiments. Work associated with SARS-CoV-2 was undertaken in the ABSL-3 facilities of the UVA, and experiments involving influenza were executed in the ABSL-2 facilities of the UVA.

[0072] *Mouse.* Mice of the following strains were procured from the Jackson Laboratory: WT C57BL/6 (catalog no. 000664), Cd11c-Cre (catalog no. 008068), Lyz2-Cre (catalog no. 004781), *Pex5*^{fl/fl} (catalog no. 031665), and SPC-EGFP (catalog no. 028356). They were subsequently bred in-house. *Pex5*^{ΔCd11c} and *Pex5*^{ΔLyz2} strains were established by crossing *Pex5*^{fl/fl} mice with either Cd11c-Cre or Lyz2-Cre mice. All mice were housed under specific pathogen-free conditions.

[0073] *Viruses.* Influenza A/PR8/34 virus was grown in the allantoic cavity of 10-day-embryonated hen's eggs and were free of bacterial, mycoplasma and endotoxin contamination. The isolated viruses were stored at -80 °C and titrated on Madin-Darby canine kidney cells. The SARS-CoV-2 mouse-adapted strains, MA30 and MA10, were generously provided by Dr. Stanley Perlman (University of Iowa) and Dr. Barbara J. Mann (University of Virginia School of Medicine), respectively. These strains were cultured in Vero E6 cells (ATCC CRL-1587), with the viral titer determined via a plaque assay using Vero E6 cells.

[0074] *Infection.* WT C57BL/6 mice, aged 8-12 weeks, were subjected to various doses of IAV infection, categorized as none (control group), mild (5 PFU), severe (150 PFU), and lethal (250 PFU). Similarly aged $Pex5^{ACd11c}$ or $Pex5^{fl/fl}$ mice were subjected to IAV doses of 150 PFU for morbidity studies or 250 PFU for mortality assessments. Mice of the $Pex5^{\Delta Lyz2}$, $Pex5^{fl/fl}$, $Cd11c^{cre}Pex5^{fl/wt}$, or $Pex5^{fl/fl}$ strains, aged 8-12 weeks, were infected with 150 PFU IAV for morbidity experiments.

[0075] For SARS-CoV-2 MA10 exposure, $Pex5^{ACd11c}$ or $Pex5^{fl/fl}$ mice within the 8-12 week age range received a 10^5 PFU dose to examine morbidity.

[0076] A separate cohort of older male C57BL/6 mice aged 5 to 6 months, was used for SARS-CoV-2 MA30 infection studies, based on previous work⁴⁰ and our own lab titration. These mice underwent varied infection intensities: none, mild (10 PFU), severe (2000 PFU), and lethal (5000 PFU). Within the same age bracket, $Pex5^{ACd11c}$ or $Pex5^{fl/fl}$ males were administered either 1000 PFU or 2000 PFU for morbidity and mortality studies, respectively.

For evaluating chronic sequelae, $Pex5^{ACd11c}$ or $Pex5^{fl/fl}$ mice (8-12 weeks old) were infected with 150 PFU IAV, while those aged 5 to 6 months male mice received 1000 PFU of SARS-CoV-2 MA30.

[0077] All infections were administered intranasally under anesthesia. Mice were initially weighed to establish a baseline, and daily subsequent weigh-ins were consistently performed. Clinical endpoints were identified upon a weight loss of 30% relative to their original weight.

[0078] *In vivo treatments.* WT C57BL/6 mice (8-12 weeks old) were administered 1 mg anti-IFN γ , anti-IFN α 1 or isotype control immunoglobulin G (IgG) in 200 μ L of phosphate-buffered saline (PBS) intraperitoneally on day 1 after 250 PFU IAV infection. WT C57BL/6 mice (8-12 weeks old) were administered DMSO as vehicle or 300 mg/kg 4-PBA (Sigma P21005) in 200 μ L PBS from day 4 to 8 post 250 PFU IAV infection. WT C57BL/6 male mice (18-26 weeks old) were administered DMSO as vehicle or 300 mg/kg 4-PBA in 200 μ L PBS from day 1 to 6 after 5000 PFU SARS-CoV-2 MA30 infection.

[0079] *Human lung tissue specimens.* Human lung samples were obtained from patients enrolled in the IRB-approved Lung Institute BioBank (LIBB) study at Cedars-Sinai Medical Center, Los Angeles, CA. All participants or their legal representatives provided informed written consent. Lung tissues were processed within 24 hours after surgical removal. Specifically, the lung tissues were cut and immediately fixed in 10% normal-buffered formalin for 24 hours before tissue processing using the HistoCore PEARL – Tissue Processor, Leica Biosystem, Deer Park, IL, and embedded in paraffin for histological studies. The formalin-

fixed paraffin-embedded cassettes were properly stored at room temperature until further sectioning. The lung sections were performed H&E stain, Masson's trichrome stain and immunofluorescence studies.

[0080] *Human AMs culture and treatment in vitro.* For human AMs, we selected donors without a history of immunosuppression and chemo- or radiotherapies and free of inflammation or pulmonary infection. All participants provided written informed consent before sample collection and subsequent analysis. Human AMs were obtained from BAL of adult donors undergoing flexible bronchoscopy as described before⁵⁷. About 100 to 200 ml of saline were instilled in 20-ml aliquots until 60 ml of lavage fluid were obtained. The specimen was placed on ice and immediately hand-carried to the laboratory for cell isolation. AMs were purified by adherence on coverslip (Thomas Scientific, catalog no. 64-0712) for 2 hours in complete medium (RPMI 1640, 10% FBS, and 1% penicillin/streptomycin/glutamate) at 37°C and 5% CO₂. The nonadherent cells were washed off with warm PBS. The remaining adherent cells were cultured overnight in complete medium supplemented with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (50 ng/ml) (BioLegend, catalog no. 572903) and M-CSF (BioLegend, catalog no. 574804). The following day, human AMs were stimulated with or without recombinant 50 ng/mL IFN γ (Peprotech, AF-300-02) for 24 hours, post which they were subjected to IF analysis. For the 4-PBA treatment assay, in parallel to IFN γ stimulation, AMs were treated with DMSO as vehicle or 4-PBA (1 mM) for 24 hours.

[0081] *Flow cytometry.* Single cell suspensions were preincubated with anti-Fc γ RIII/II (Fc block) before 30 min of incubation with appropriate fluorochrome-labelled antibodies. The following antibodies (supplied by BioLegend or BD Biosciences) and staining reagents were used: CD45-PerCP/Cy5.5 (BioLegend, clone 30-F11, catalog no. 103132), SiglecF-BV421 (BD Biosciences, clone E50-2440, catalog no. 562681), CD11b-FITC (BioLegend, clone M1/70, catalog no. 101206), CD11c-BV510 (BioLegend, clone N418, catalog no. 117338), Ly6G-PE/Cy7 (BioLegend, clone 1A8, catalog no. 127618), Ly6C-BV711 (BioLegend, clone HK1.4, catalog no. 128037), CD64-PE (BioLegend, clone X54-5/7.1, catalog no. 139304), MerTK-APC (BioLegend, clone 2B10C42, catalog no. 151508), CD4-BV785 (BioLegend, clone RM4-5, catalog no. 100551), CD8-BV421 (BioLegend, clone 53-6.7, catalog no. 100737), CD44-BV510 (BioLegend, clone IM7, catalog no. 103043), influenza NP₃₆₆₋₃₇₄ tetramer [NIH Tetramer Facility, catalog no. H-2D(b) ASNENMETM], influenza PA₂₂₄₋₂₃₃ tetramer [NIH Tetramer Facility, catalog no. H-2D(b) SLENFRAYV], and SARS-CoV-2 Spike₅₃₉₋₅₄₆ tetramer [NIH Tetramer Facility, catalog no. H-2K(b) VNFNFNGL]. The dilution

of surface staining Abs was 1:300. Staining samples were analyzed on a FACS Attune or FACS Attune NXT flow cytometer (Life Technologies) and interpreted using the software FlowJo (Treestar).

[0082] *Mouse AMs culture and treatment in vitro.* Mouse AMs were obtained from BALF as described previously. (Huang, S. *et al.* PPAR-gamma in Macrophages Limits Pulmonary Inflammation and Promotes Host Recovery following Respiratory Viral Infection. *J. Virol.* (2019) 93, doi:10.1128/JVI.00030-19.)

[0083] For the naive mice, AMs were purified by adherence for 2 hours in complete medium (RPMI 1640, 10% FBS, and 1% penicillin/streptomycin/glutamate) at 37°C and 5% CO₂. The nonadherent cells were washed off with warm PBS. The adherent AMs were cultured in complete medium supplemented with recombinant murine GM-CSF (10 ng/ml) (BioLegend, catalog no. 576304). For *in vitro* treatment, AMs were stimulated with the following concentrations of cytokines where indicated unless otherwise noted: 50 ng/mL of R848 (Invivogen, catalog no. tlr-r848), 5 µg/mL of CpG (Invivogen, catalog no. tlr-1826), 20 ng/mL of LPS (Invivogen, catalog no. tlr-eklps), 20 ng/mL of Il-6 (Biolegend, catalog no. 575704), 20 ng/mL of Il-1b (Biolegend, catalog no. 575104), 25 ng/mL of TNFa (peprotech, catalog no. 315-01A), 50 ng/mL of IFNg (peprotech, catalog no. 3 315-05), 50 ng/mL of IFNa (Biolegend, catalog no. 752804), 50 ng/mL of IFNl (Biolegend, catalog no. 575304), or 5 µg/mL of Poly(I:C) (Invivogen, catalog no. tlr-pic). For Mitochondrial ROS inhibitor treatment, AMs were treated with DMSO (vehicle) or Mito-TEMPO (100 µM), and concurrently exposed or not exposed to Poly(I:C) for 24 hours. In the case of the 4-PBA treatment assay, except IFNg stimulation, simultaneously, AMs also treated with DMSO (vehicle) or 4-PBA (1 mM) for 24 hours. ~

[0084] *Cell Immunofluorescence.* For *ex vivo* experiments, BALF cell suspensions were stained with the appropriate Ab cocktail in flow cytometry buffer at 4°C for 30 min. AMs (CD11c⁺ Siglec F⁺ CD11b^{low} CD64⁺ MerTK⁺) were sorted using the BD Influx cell sorter and adhered for 2 hours on coverslips (Thomas Scientific, catalog no. 1139W09). For *in vitro* experiments, AMs were cultured and treated on coverslips. Cells were fixed using 4% paraformaldehyde in PBS for 10 min. Afterward, they were washed in PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Subsequently, cells were incubated with the primary antibody in Agilent Dako antibody dilute solution (S302283) overnight at 4°C. Following PBS washes, secondary antibodies (1:500) were applied and incubated for 1 hour at room temperature. Coverslips were then mounted onto glass microscope slides using ProLong

Gold Antifade Mountant with DAPI (Invitrogen). The following antibodies were used: Mouse anti-PMP70 (Sigma, SAB4200181, 1:200), Rabbit anti-LC3B (abcam, ab192890, 1:200), Goat anti-mouse IgG AF555 (ThermoFisher, A32727), DyLight 649 Donkey anti-rabbit IgG (BioLegend, 406406).

[0085] *Lung immunofluorescence.* Lung tissue sections (5µm) were deparaffinized in xylene and rehydrated. For antigen retrieval, heat was applied using the 1X Agilent Dako target retrieval solution (pH 9) (S236784) or a sodium citrate buffer (10mM Sodium Citrate, 0.05% Tween 20, pH of 6.0). The retrieval process in a steamer took 20 minutes for mouse lungs and 45 minutes for human lungs. This was followed by both blocking and surface staining procedures. For intracellular targets, tissues were permeabilized with 0.5% Triton-X 0.05% Tween20 for 1 hour at room temperature. Sections were stained with primary antibodies overnight at 4°C. Subsequently, samples were washed and incubated with fluorescent secondary antibodies for 2 hours at room temperature. Sections were counterstained with DAPI (1:1000, ThermoFisher Scientific) for 3 minutes and mounted using ProLong Diamond Antifade mountant (ThermoFisher Scientific). After 24 hours of curing at room temperature, images were acquired using the Olympus BX63 fluorescent microscope and pseudocolours were assigned for visualization. All images were further processed using ImageJ Fiji, OlyVIA, and/or QuPath software. The following antibodies (antigen retrieval was performed using PH 9.0 unless otherwise indicated) were used: Mouse anti-CD68 (abcam, ab955, 1:150, PH 6.0), Rabbit anti-PEX14 (proteintech, 10594-1-AP, 1:200, PH 6.0), Mouse anti-CD169 (Invitrogen, MA1-16891, 1:50), Mouse anti-pro-SPC (Santa Cruz, sc-518029, 1:200), Rabbit anti-pro-SPC (Sigma, AB3786, 1:500), Rabbit anti-AGER (proteintech, 16346-1-AP, 1:200), Hamster anti-PDPN (abcam, ab11936, 1:500), Rat anti-Ki-67 (Invitrogen, 14-5698-82, 1:100), Chicken anti-cytokeratin 5 (Biolegend, 905901, 1:200), Chicken anti-cytokeratin 5 (Biolegend, 905903, 1:200), Rat anti-cytokeratin 8 (TROMA-1) (DSHB, Antibody Registry ID: AB_531826, 1:300), DyLight 649 Donkey anti-rabbit IgG (1:125), Goat anti-mouse IgG AF488 (ThermoFisher, A-11001, 1:300), Goat anti-rat IgG AF555 (ThermoFisher, A48270, 1:300), Goat anti-Armenian Hamster IgG AF488 (ThermoFisher, A78963, 1:400), Goat anti-Chicken IgY AF750 (abcam, ab175755, 1:100), Goat anti-rat IgG AF647 (ThermoFisher, A-21247, 1:200), Goat anti-rabbit IgG AF488 (ThermoFisher, A-11008, 1:300).

[0086] *AT2 cell isolation and culture.* AT2 cells were isolated from WT C57BL/6 mice as previously described. (See Weiner, A. I. *et al.* (2022) *Cell Rep* 41, 111805, doi:10.1016/j.celrep.2022.111805, Major, J. *et al.* (2023) *Nature* 621, 813-820,

doi:10.1038/s41586-023-06287-y, and Riemondy, K. A. *et al.*, (2019) *JCI Insight* 5, doi:10.1172/jci.insight.123637.) Briefly, mouse lungs were perfused with chilled PBS and intratracheally instilled with 1mL of dispase II (15U/mL, Roche), tying off the trachea and cutting away the lobes from the mainstem bronchi. Lungs were incubated in 4mL of 15U/mL dispase II for 45min while shaking at room temperature, followed by mechanical dissociation with an 18G needle. Following passage through a 100µm filter, lungs underwent 10min of DNase I digestion (50µg/mL) and filtered through 70µm filter prior to RBC lysis. Single-cell suspensions were subject to CD45 depletion using microbeads (Miltenyi), incubated with anti-FcγRIII/II (Fc block) and stained with CD45, EpCAM, MHC-II, and viability dye.

[0087] Fluorescence assisted cell sorting was performed on the BD Influx cell sorter to isolate AT2 cells as described previously (36) and collected in 500µL DMEM + 20% FBS + 2% P/S. Sorted AT2 cells (2×10^5 /well) were plated in a 96-well plate in DMEM/F12 + 10% FBS and cultured at 37°C, 5% CO₂ for 3 days prior to harvest.

[0088] *Lipidomic analyses.* For each sample, 2 million AMs or BMDMs isolated from *Pex5^{ΔLys2}* or *Pex5^{fl/fl}* mice were treated with Poly(I:C) *in vitro* for 24 hours. The following day, the adherent cells were gently scraped off and transferred to 1.5 ml conical tubes. After centrifugation to pellet the cells, the supernatant (culture medium) was carefully removed. The cell pellet was washed once with ice-cold PBS and then quickly submerged in liquid nitrogen for 10 minutes to snap freeze. Subsequently, the samples were stored at -80°C until analysis. Metabolomic analysis of the samples was conducted by the metabolomics core at UVA. Data were analyzed with Metaboanalyst 5.0, lipid ontology enrichment analysis was performed using LION/web tool.

[0089] *Seahorse analyses.* Cellular oxygen consumption rate was measured using Agilent Seahorse XFe96 Analyzer, according to manufacturer's instructions. The cell culture medium was replaced with unbuffered basic medium (pH 7.4, Agilent Technologies) with 10 mM glucose. The following compounds were added to test mitochondrial activity: 1 µM oligomycin, 1.5 µM FCCP, 0.5 µM rotenone with 0.5 µM antimycin A. Data were analyzed with Wave Desktop software version 2.6 (Agilent Technologies).

[0090] *Measurement of mitochondrial mass.* AMs or BMDMs (1×10^5) were seeded into 24-well plates and stimulated with or without Poly(I:C) (5 µg/ml) overnight at 37°C and 5% CO₂. On the following day, the cells were washed and incubated with MitoTracker Deep Red (Invitrogen, catalog no. M22426) and MitoTracker Green (Invitrogen, catalog no. M7514) at

50 nM for 30 min at 37°C. Then, cells were washed twice with PBS and lifted off the plates for flow cytometry.

[0091] RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR). RNA was isolated using Total RNA purification kit and treated with DNase I (Sigma, Cat# RTN350) according to the manufacturer's instructions. Random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Cat# 28025021) were used to synthesize first-strand cDNAs from equivalent amounts of RNA from each sample. Quantitative PCR with reverse transcription (RT-qPCR) was performed with Fast SYBR Green PCR Master Mix (Applied Biosystems, Cat# 4385618). qRT-PCR was conducted in duplicates in QuantStudio 6 Flex (Applied Biosystems). Data were generated with the comparative threshold cycle (Delta CT) method by normalizing to hypoxanthine phosphoribosyltransferase (HPRT).

[0092] *Bulk RNA sequencing.* Total RNA from *in vitro* cultured AMs was used for bulk RNA-seq. After quality control, high-quality (Agilent Bioanalyzer RIN >7.0) total RNA was used to generate the RNA-seq library. cDNA synthesis, end-repair, A-base addition, and ligation of the Illumina indexed adapters were performed according to TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA). The concentration and size distribution of the completed libraries were determined using an Agilent Bioanalyzer DNA 1000 chip (Santa Clara, CA) and Qubit fluorometry (Invitrogen, Carlsbad, CA). Paired-end libraries were sequenced on Illumina HiSeq 4000 following Illumina's standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit. Base calling was performed using Illumina's RTA software (version 2.5.2). Paired-end RNA-seq reads were aligned to the mouse reference genome (GRCm38/mm10) using RNA-seq spliced read mapper Tophat2 (v2.2.1). Pre- and post-alignment quality controls, gene-level raw read count, and normalized read count [i.e., fragments per kilobase per million reads (FPKM)] were performed using the RSeQC package (v2.3.6) with the National Center for Biotechnology Information (NCBI) mouse RefSeq gene model. Differential expression for each gene between various groups specified in the text was identified on basis of the results of DESeq2 Wald tests. For visualization, data were logarithmic-transformed, and genes that exhibited log2 fold change values > 2 and log10 P > 25 between compared groups were highlighted. For functional analysis, gene set enrichment analysis (GSEA) was applied to identify enriched gene sets from MSigDB, using a weighted enrichment statistic and a log2 ratio metric for ranking genes. The bulk RNA-seq was conducted once using multiple biological samples per group (as indicated in figures).

[0093] *Single-cell RNA sequencing.* *Pex5*^{ΔCd11c} or *Pex5*^{fl/fl} mice were either uninfected (Day 0) or infected with 150 PFU of IAV. Lung cells were harvested on day 0 and day 8 and 14 post infection. For each time point, cells from three individual mice per genotype were pooled and subjected to single-cell RNA sequencing (scRNA-seq) analysis. For library preparation, we utilized the Chromium Single Cell 3' Reagent Kit from 10x Genomics, strictly adhering to the manufacturer's guidelines. The paired-end sequencing was conducted on the DNBSEQ-G400 platform in its rapid-run mode. The resulting scRNA-seq data underwent alignment and quantification processes via the 10x Genomics Cell Ranger Software Suite. To ensure data purity, we employed the "scDblFinder" package to eliminate any doublet cells. The curated cell data were then analyzed using the "Seurat" software (version 4.1.1). For quality assurance, cells were filtered based on the criteria: gene count > 200, UMI count > 1000, and mitochondrial gene content < 5%. Our analytical workflow encompassed normalization, dimensionality reduction, clustering, and the identification of marker genes for clusters and differentially expressed genes. Further downstream analysis, such as GSEA, was conducted based on the results derived from the FindAllMarkers function, employing the clusterProfiler package. (Yu, G., et al., (2012) *OMICS* 16, 284-287, doi:10.1089/omi.2011.0118.)

[0094] *BCA protein assay.* BCA protein assay kit was obtained from Thermo Scientific (Cat# 23225). 2 μl of each BAL sample was used. VERSAmax microplate reader (Molecular Devices) was used for colorimetric quantification and analysis at 570nm wavelength.

[0095] *Virus titer measurement.* For IAV, the viral titers in the BAL were measured by endpoint dilution assay and expressed them as tissue culture infectious dose 50 per (TCID₅₀). Briefly, MadinDarby canine kidney cells (MDCK, The American Type Culture Collection) were grown in 96- well plates and incubated with 10-fold dilutions of BAL sample from IAV-infected mice with different treatments in serum-free DMEM medium. After a 3-day incubation at 37 °C in a humidified atmosphere of 5% CO₂, the supernatants were collected and mixed with a half volume of 0.5% chicken red blood cells (Rockland, Cat# R401-0100). After 30 min incubation at room temperature (RT), the agglutination pattern was analyzed and the TCID₅₀ values were calculated.

[0096] For SARS-CoV-2 MA10, the viral titer in the BAL were determined using plaque assay. Vero E6 cells were cultured in DMEM with the addition of 2% Fetal Clone II serum (Hyclone) and 1% Pen/Strep/glutamate. Serial dilutions were added to the cells. The plate was incubated at 37°C and 5% CO₂ for 1 hour, shaking the plates every 15 minutes. After incubation, monolayers were overlaid with media containing 1.2% Avicel PH-101 and

incubated at 37 °C and 5% CO₂. After 72 hours, the overlay was removed, wells were fixed with 10% formaldehyde, and stained with 0.1% crystal violet to visualize plaques. Plaques were counted, and PFUs were calculated according to the following equation: Average # of plaques/dilution factor × volume diluted virus added to the well.

[0097] *BMDMs culture and treatment in vitro.* BMDMs were isolated from bone marrow of *Pex5^{fl/fl}* or *Pex5^{ΔLyz2}* mice. The bone marrow cavities were repeatedly flushed with DMEM containing 10% FBS. After red cells were removed, the remained cells were cultured in DMEM supplemented with 50 ng/mL of M-CSF (Biolegend, Cat# 576404), 10% FBS and 1% Pen/Strep/glutamate at 37 °C and 5% CO₂ for 7 days with medium renewed at day 4. BMDMs were stimulated with or without 5 μg/mL Poly(I:C).

[0098] *Immunoprecipitation and Immunoblots.* Cells were lysed in RIPA buffer (Sigma, CAT# R0278) with a protease inhibitor cocktail (Sigma, CAT# P8340) and 4x sample loading buffer (containing SDS and 2-mercaptoethanol). Proteins were separated by electrophoresis through 4% -12% SDS-PAGE. For Immunoprecipitation assay, AMs were stimulated with or without IFNγ overnight *in vitro*. DMSO or MG132 (0.5 μM) were added 6 h before cell harvesting. Cells were lysed in RIPA buffer for 30 minutes on ice. The BCA protein assay kit was used to determine protein concentrations. From each sample, an equal amount of protein was isolated and immunoprecipitated using the anti-PMP70 antibody. Subsequently, the samples underwent Western blotting analysis. The antibodies used in immunoblots and co-immunoprecipitations were as follows: PEX5 (Cell signaling technology, 83020, 1:1000), PMP70 (Sigma, SAB4200181, 1:1000), PEX14 (proteintech, 10594-1-AP, 1:1000), Ubiquitin (E6K4Y) (Cell signaling technology, 20326, 1:1000), α-SMA (abcam, ab7817, 1:1000), Collagen I (abcam, ab270993, 1:1000), GAPDH (Cell Signaling Technology, Cat# 97166S, 1:2000).

[0099] *Scratch-wound assay.* AMs from *Pex5^{fl/fl}* or *Pex5^{ΔCd11c}* mice underwent overnight incubation with either Poly(I:C), Mito-TEMPO treatments, or without any treatment. Concurrently, 10⁵ MLE-12 cells were seeded into 24-well plates. Upon reaching confluence the following day, cells were wounded using a 1,000 μl pipette tip. Post-wounding, cells were rinsed with PBS and subsequently co-cultured with the treated AMs. Wound images were captured at the initial time point (0h) and after 24h using a light microscope, ensuring consistent positioning with a marker for reference. Wound closure rates were quantified via QuPath software. The formula utilized for calculating the percentage of wound closure was: % wound

confluence = $(a - b) \times 100\% / a$, where 'a' represents the initial wound area at 0h, and 'b' denotes the area at 24h.

[0100] *Primary 3D Lung organoid co-culture.* Lung organoids were established following the previous report^{94,95}. Briefly, 3, 000 freshly sorted GFP-labeled AT2 cells (SPC-GFP) and AMs at a number 3 times of AT2s were resuspended in a 1:1 mixture of 3D basic media (DMEM/F12 [GIBCO] supplemented with 10% FBS, ITS [Insulin-Transferrin-Selenium, Corning], and TGF- β 1 inhibitor sb431542 [Sigma-Aldrich]) with growth factor-reduced Matrigel (BD Biosciences). 2×10^5 mouse lung fibroblasts, mlg2908, were added as feeder cells. A 100 mL cell mixture was placed in a 24-well Transwell insert with a 0.4-mm pore (Corning). Culture medium was added in the bottom of the wells and was refreshed every other day. Analysis of colony forming efficiency (CFE) of organoids were determined on 14 days after plating.

[0101] *Statistical analyses.* Data are means $\pm$ SEM of values from individual mice (*in vivo* experiments). Unpaired two-tailed Student's t test (two-group comparison), one-way analysis of variance (ANOVA; multiple-group comparison), multiple t test (weight loss), or log-rank test (survival study) was used to determine statistical significance by GraphPad Prism software. We considered $P < 0.05$ to be significant. * $P < 0.05$; ** $P < 0.01$. Wilcoxon rank-sum test and MAST were performed during scRNA-seq data analysis.

[0102] While at least one exemplary embodiment has been presented in the foregoing detailed description, it should be appreciated that a vast number of variations exist. It should also be appreciated that the exemplary embodiment or exemplary embodiments are only examples, and are not intended to limit the scope, applicability, or configuration of the various embodiments in any way. Rather, the foregoing detailed description will provide those skilled in the art with a convenient road map for implementing an exemplary embodiment as contemplated herein without any additional undue experimentation. It being understood that various changes may be made in the function and arrangement of elements described in an exemplary embodiment without departing from the scope of the various embodiments as set forth in the appended claims.

[0103] Since certain changes may be made in the above-described disclosure, without departing from the spirit and scope of the disclosure herein involved, it is intended that all of the subject matter of the above description shown in the accompanying drawings shall be interpreted merely as examples illustrating the inventive concept herein and shall not be construed as limiting the disclosure.

[0104] Finally, the written description uses examples to disclose the disclosure, including the best mode, and also to enable any person skilled in the art to practice the disclosure, including making and using any devices or systems and performing any incorporated methods. The patentable scope of the disclosure is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal language of the claims.

CLAIMS

1. A method of treating lung injury in a patient comprising:
administering a therapeutically effective amount of a pharmaceutical agent that enhances peroxisome biogenesis to a patient in need thereof.
2. The method of claim 1 wherein the patient is one who has an active or cleared viral respiratory infection.
3. The method of claim 2 wherein the viral respiratory infection is caused by at least one of: Sars-CoV-2, MERS, and influenza.
4. The method of claim 2 further comprising, administering at least one of an: antiviral and an anti-inflammatory.
5. The method of claim 4 wherein the antiviral is paxlovid or molnupiravir.
6. The method of claim 4 wherein the anti-inflammatory is dexamethasone or tocilizumab.
7. The method of claim 2 wherein the patient is no longer experiencing symptoms of active viral respiratory infection.
8. The method of claim 1 wherein the patient is one exposed to at least one of: caustic substances, tobacco smoke, asbestos, and fine particulate matter.
9. The method of claim 1 wherein the patient is suffering from a peroxisome biogenesis disorders.
10. The method of claim 9 wherein the peroxisome biogenesis disorder is Zellweger syndrome.
11. The method of any one of claims 1 to 9, wherein the pharmaceutical agent is 4-phenylbutyrate, a monounsaturated fatty acid, such as an omega-3 fatty acid (e.g. DHA or EPA) or an omega-6 fatty acid, or an alpha, beta, or gamma PPAR (peroxisome proliferator-activated receptor) agonist.
12. The method of claim 11 wherein the PPAR agonist is clofibrate, gemfibrozil, ciprofibrate bezafibrate, fenofibrate, pioglitazone, rosiglitazone, or lobeglitazone.

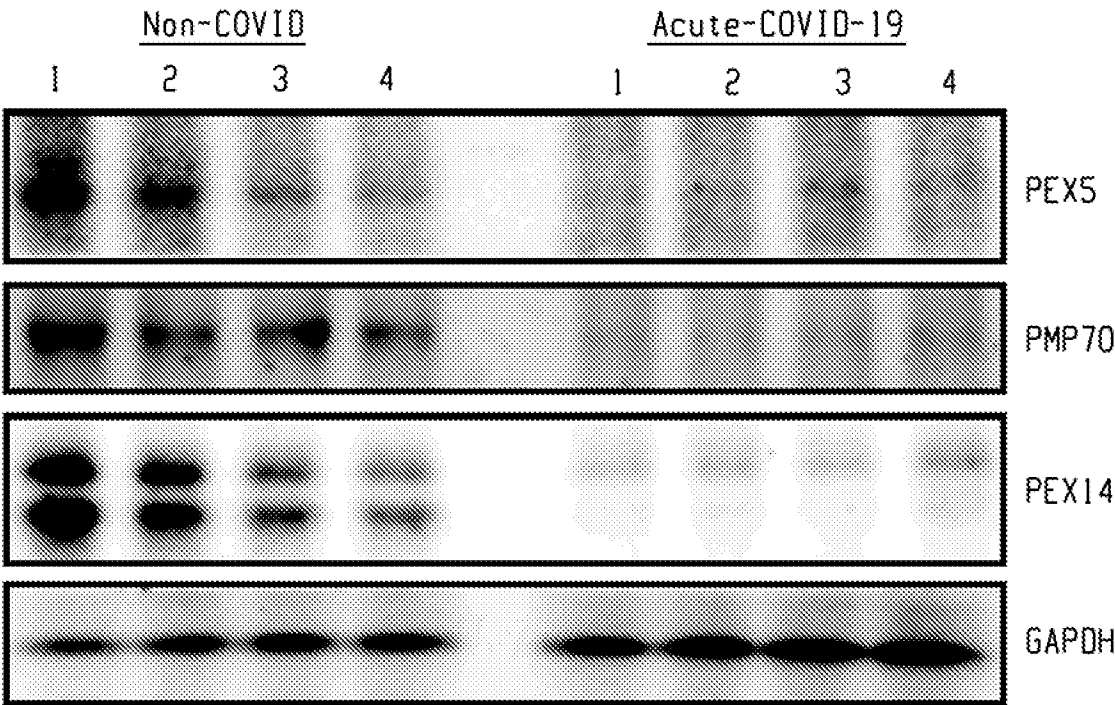


Fig. 1A

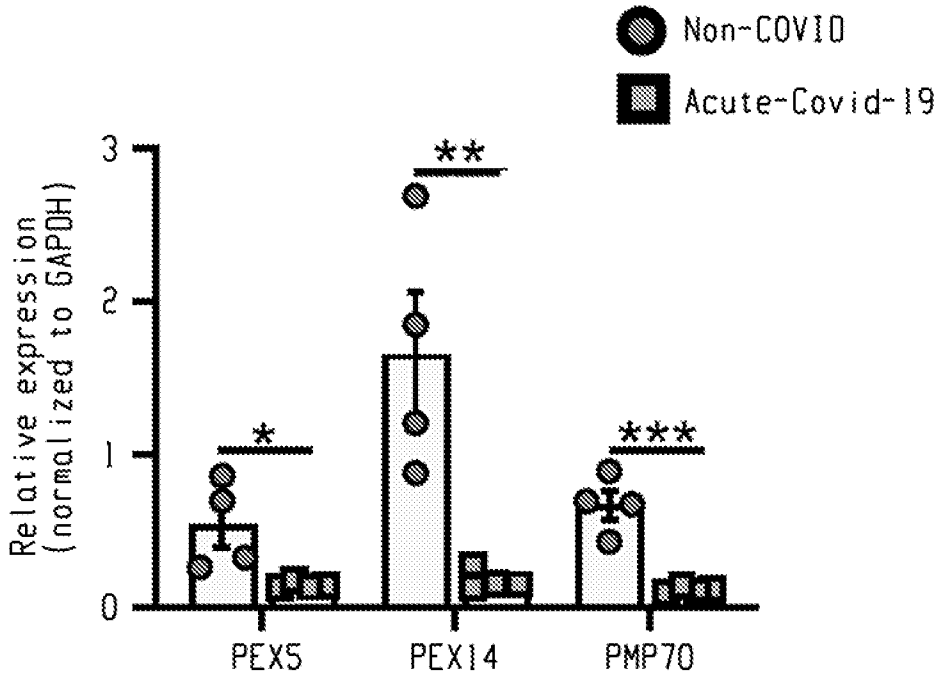
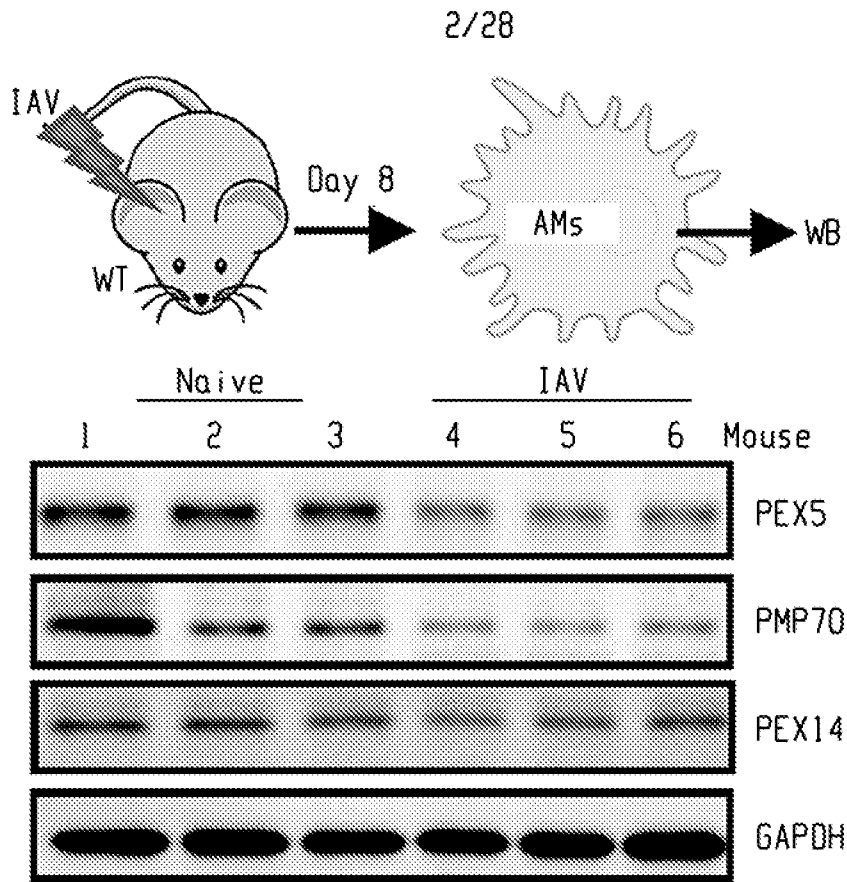
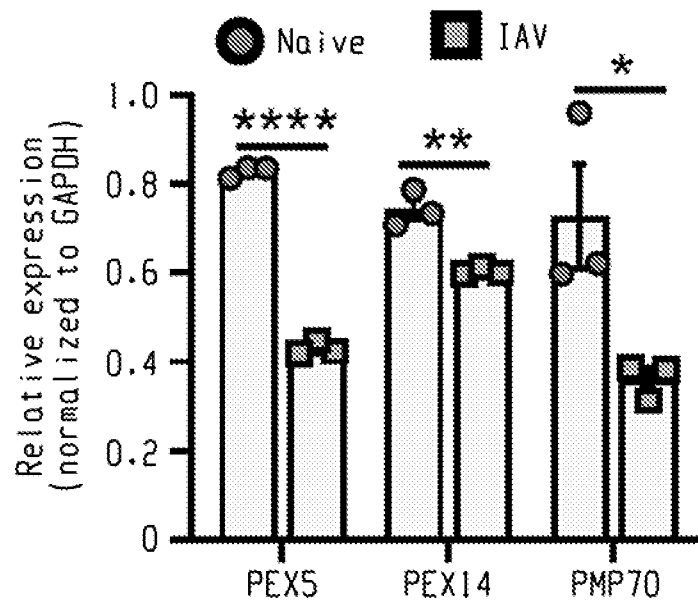


Fig. 1B

*Fig. 1C**Fig. 1D*

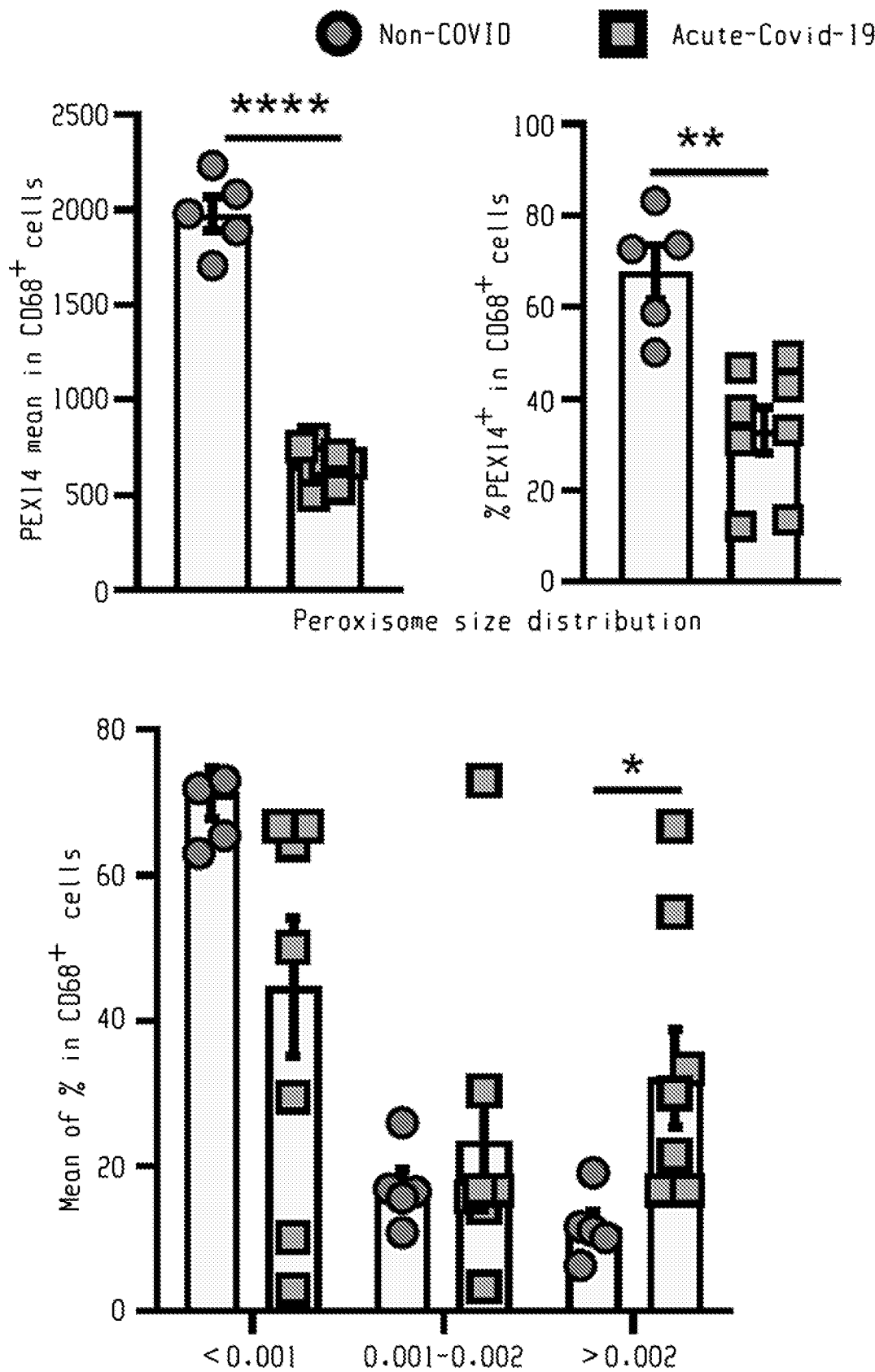


Fig. 2A

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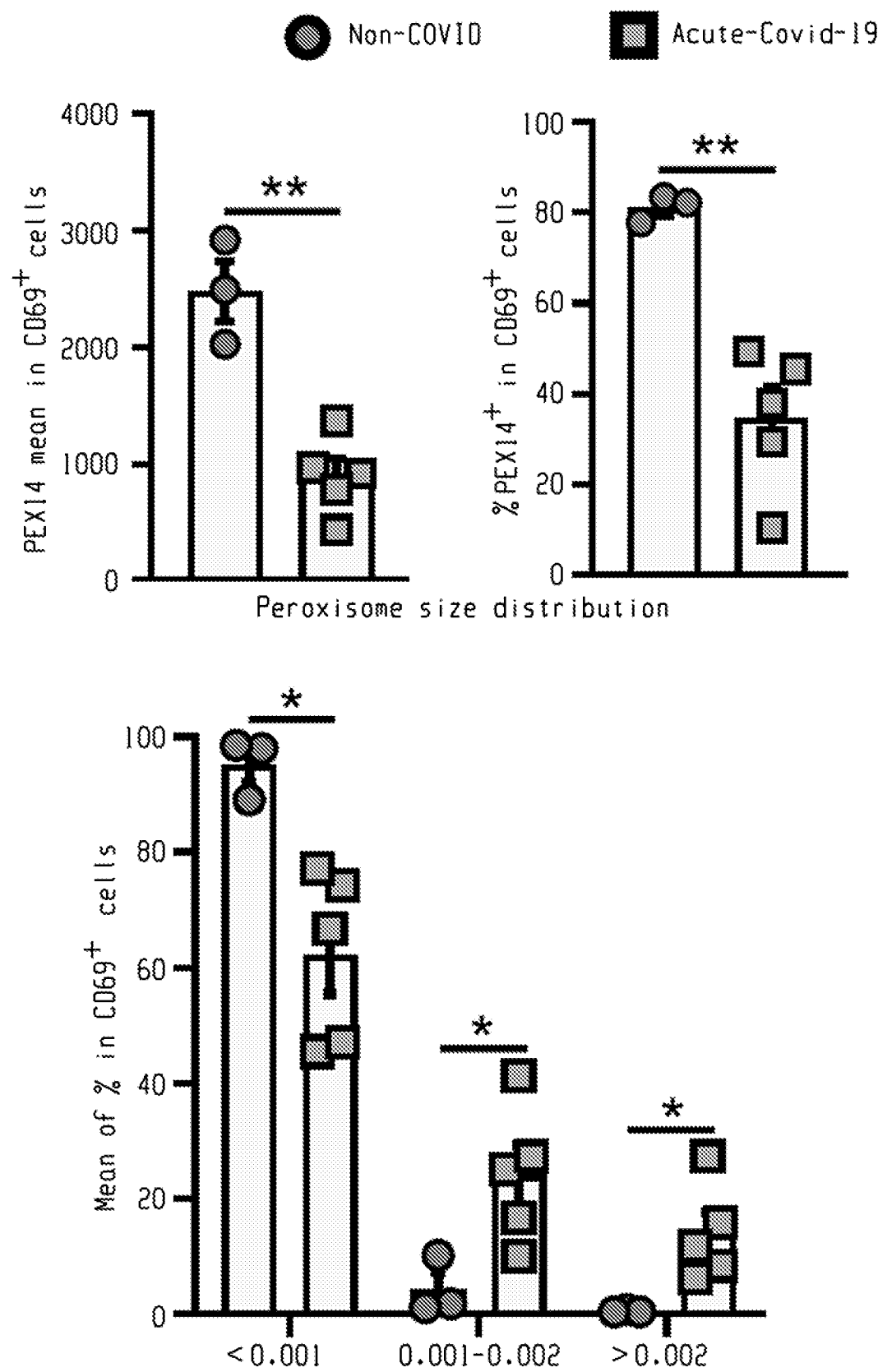


Fig. 2B

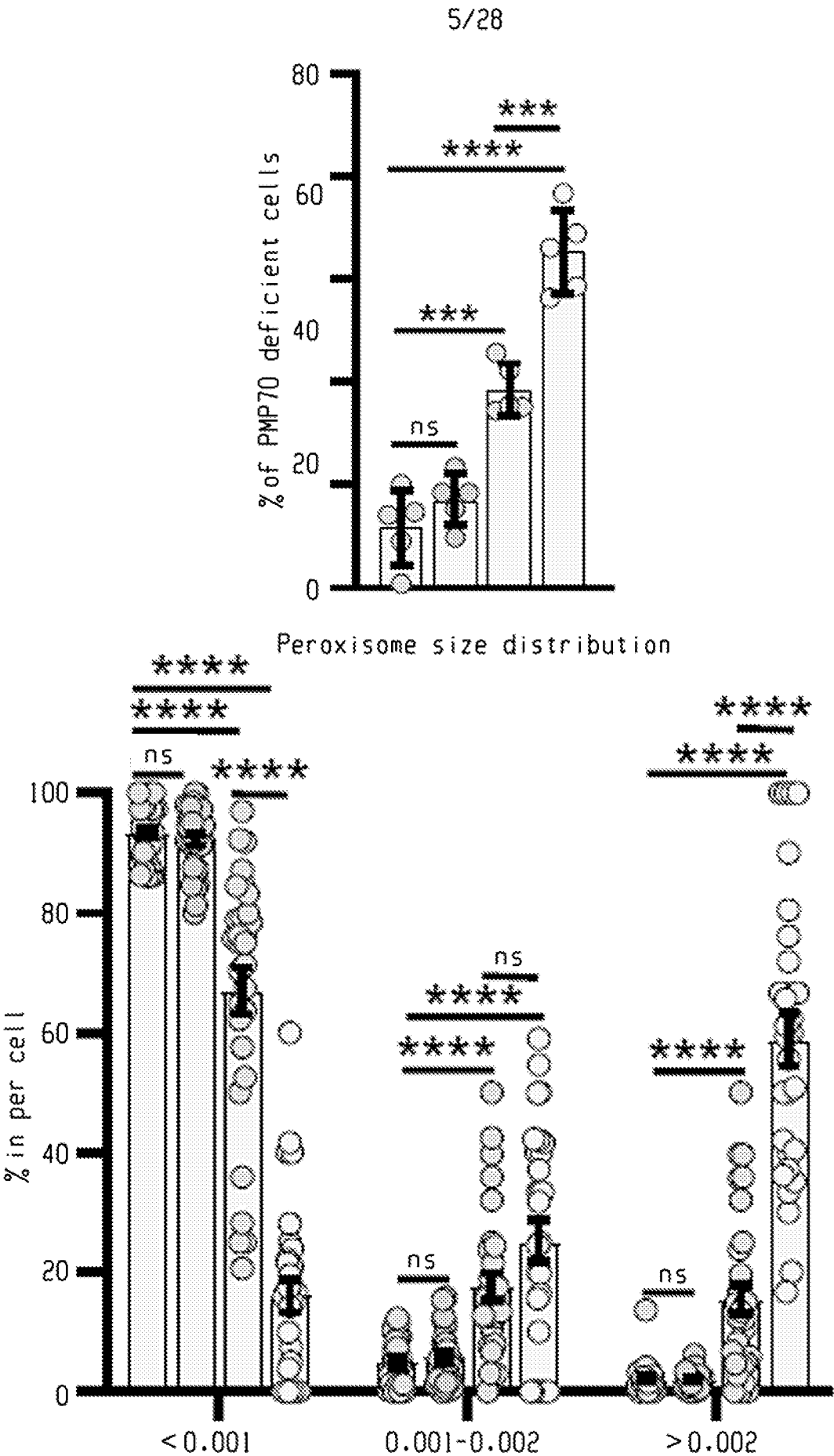


Fig. 2C

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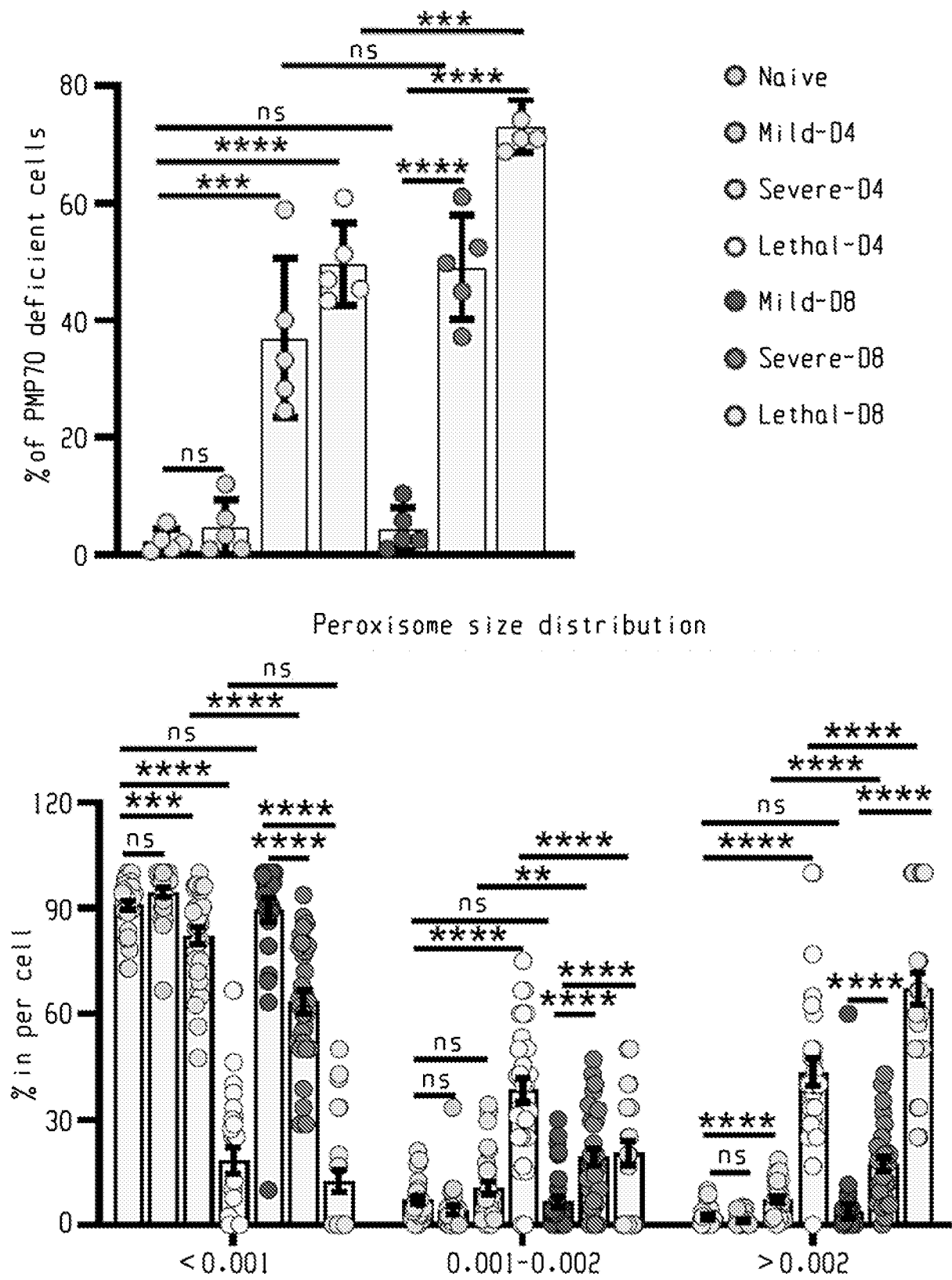


Fig. 2D

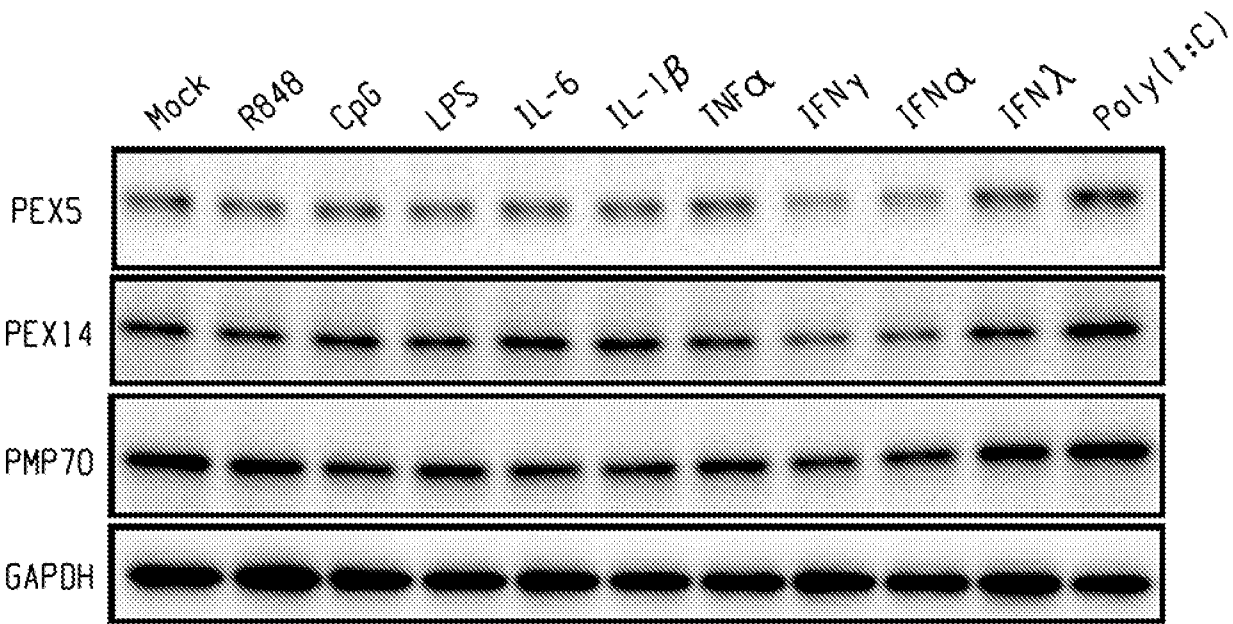


Fig. 3A

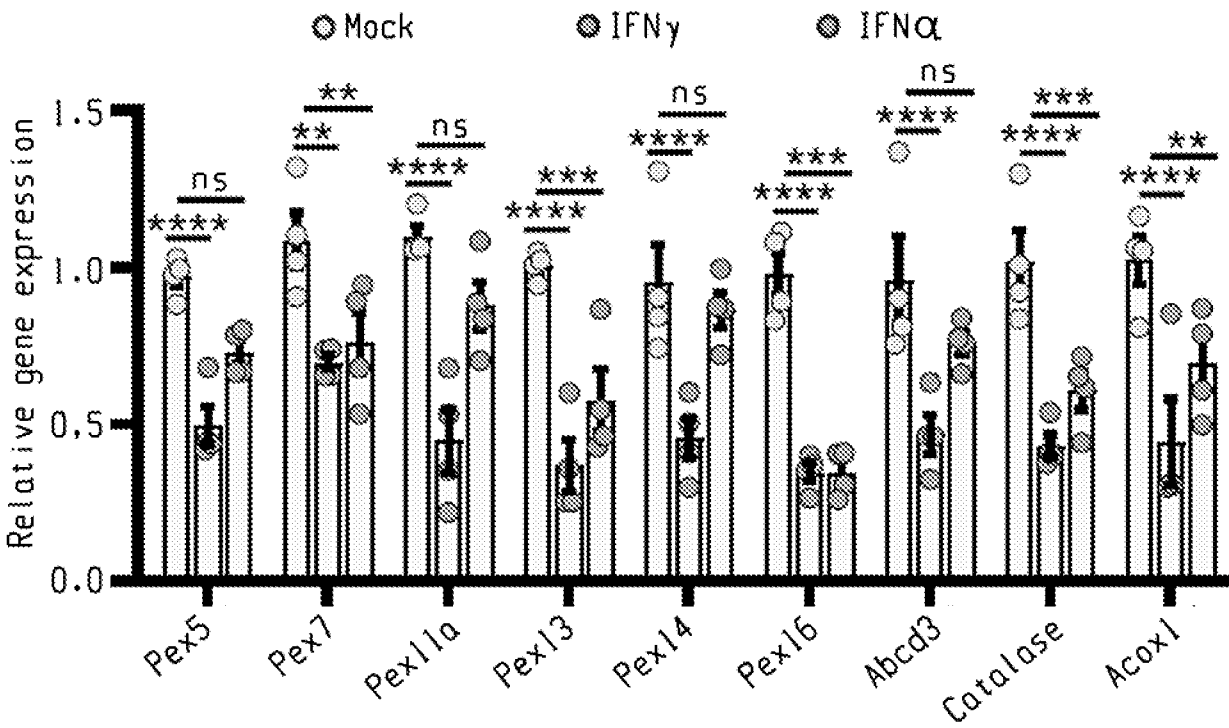


Fig. 3B

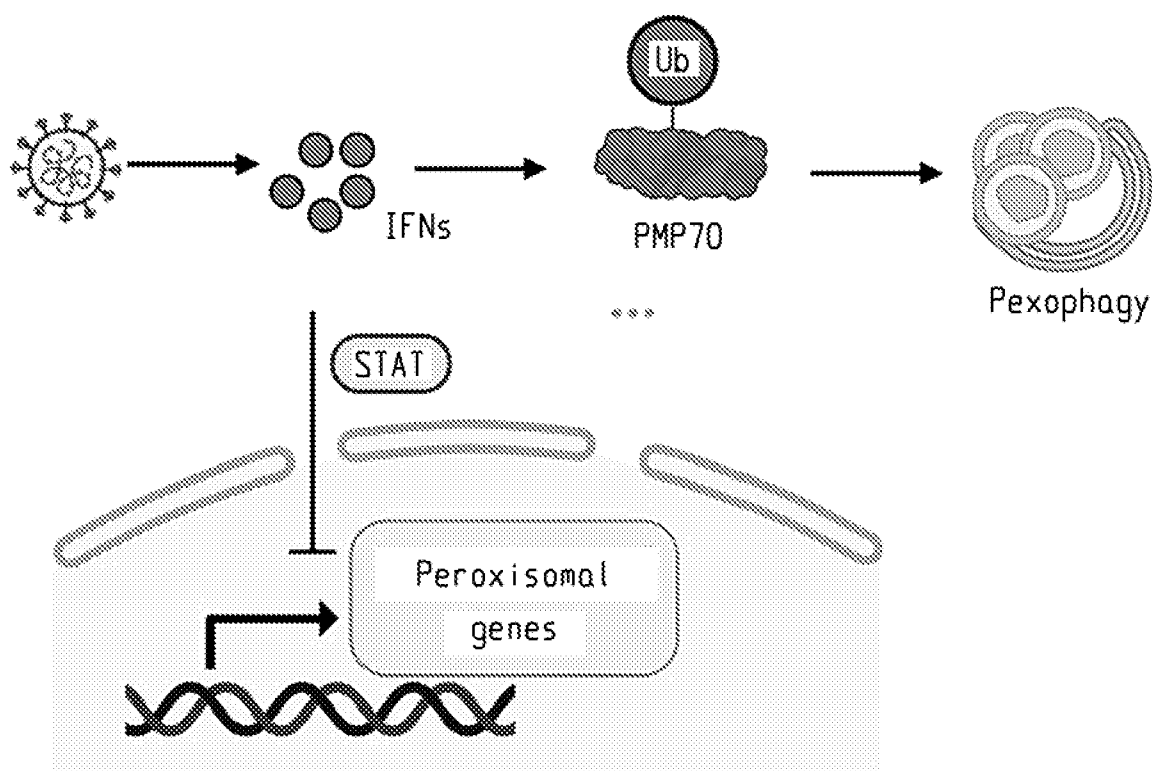


Fig. 3C

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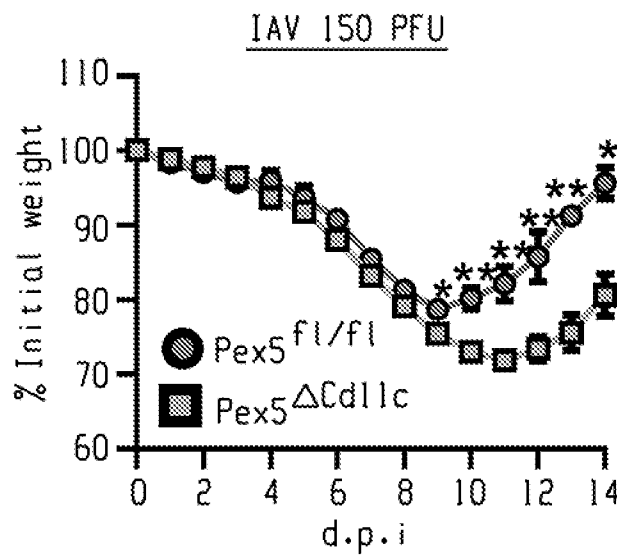


Fig. 4A

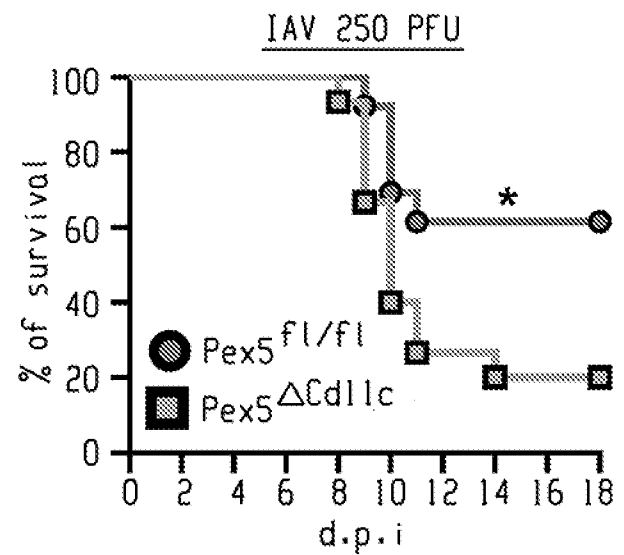


Fig. 4B

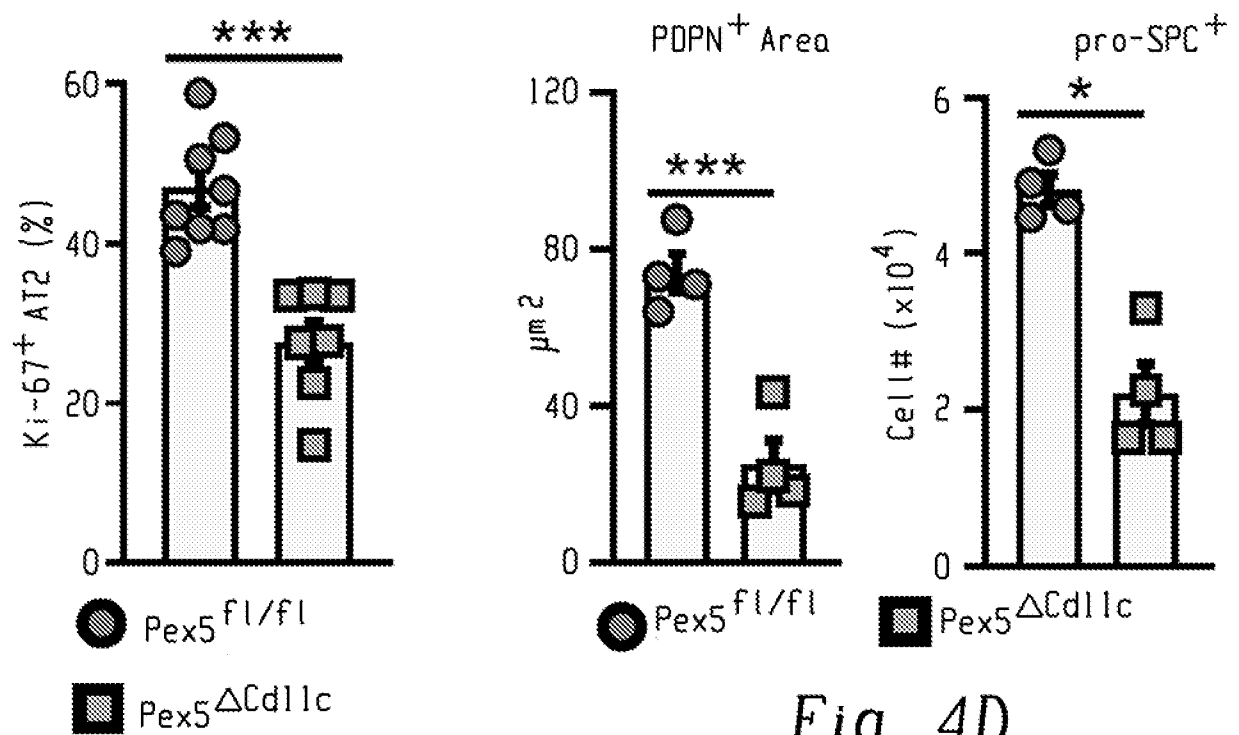


Fig. 4C

Fig. 4D

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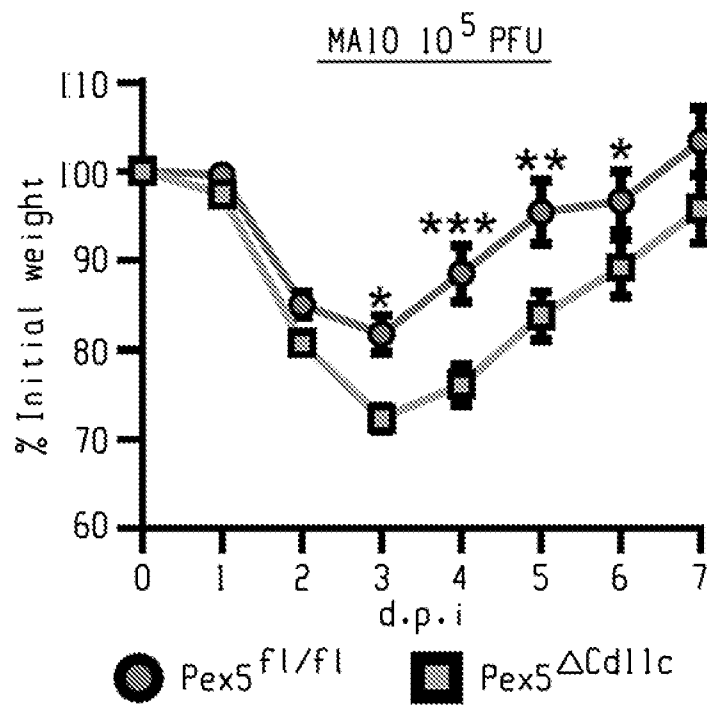


Fig. 4E

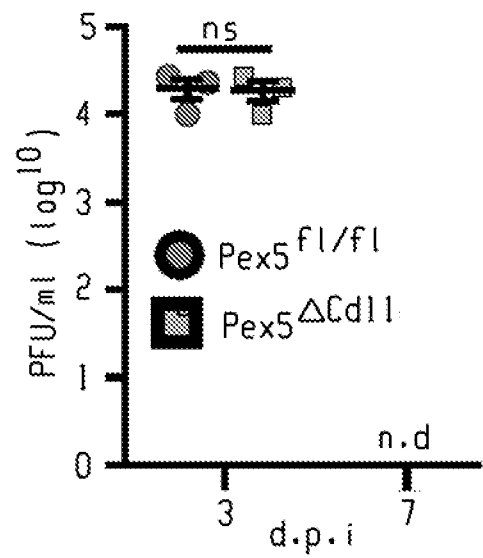


Fig. 4F

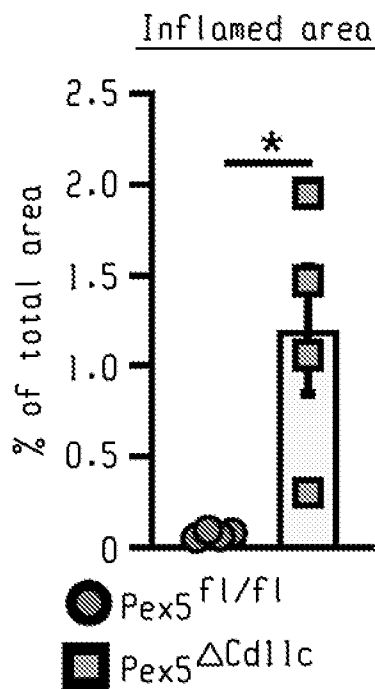


Fig. 4G

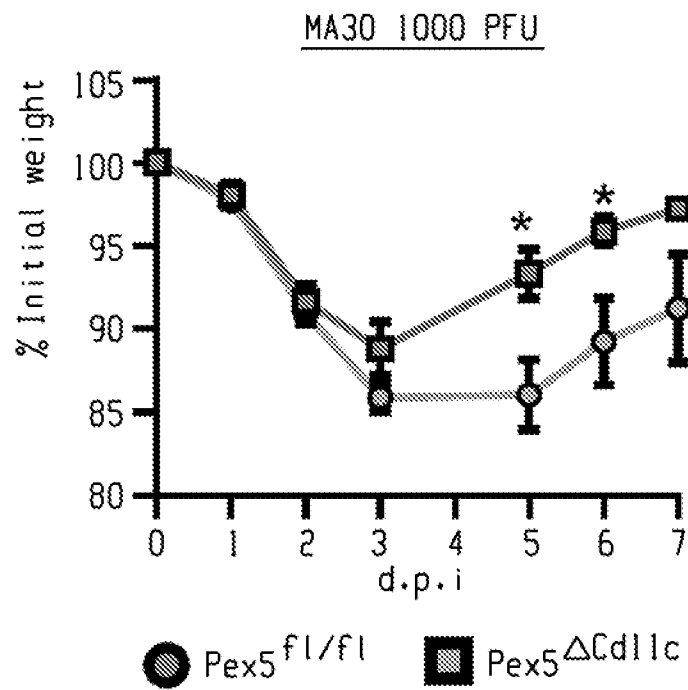
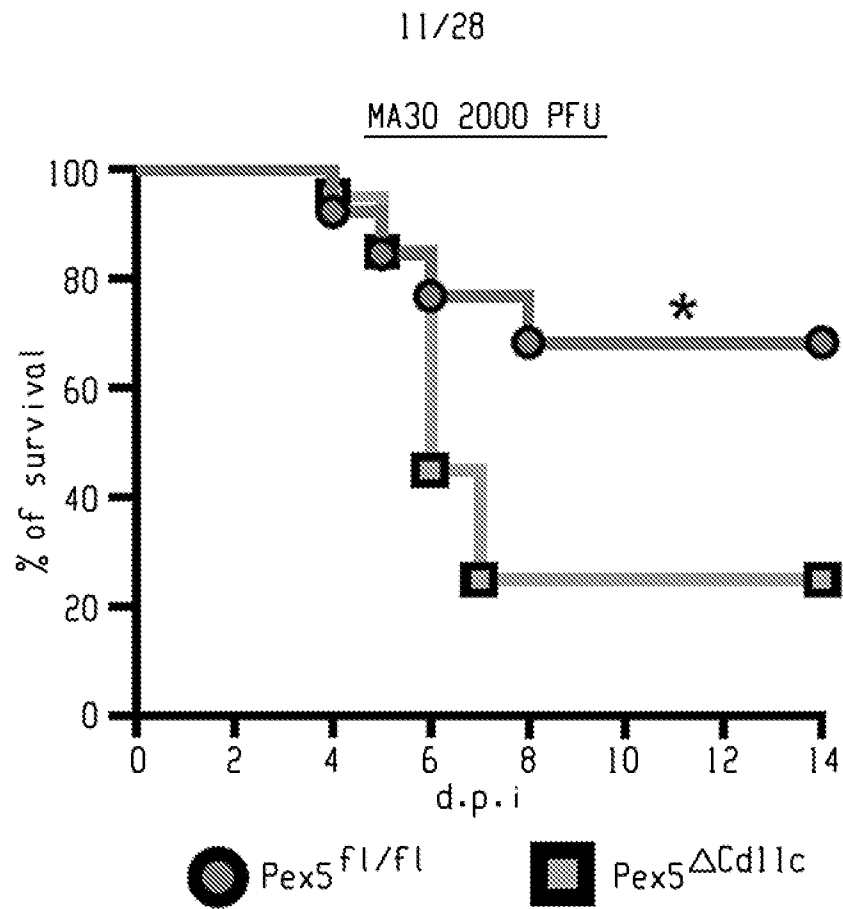
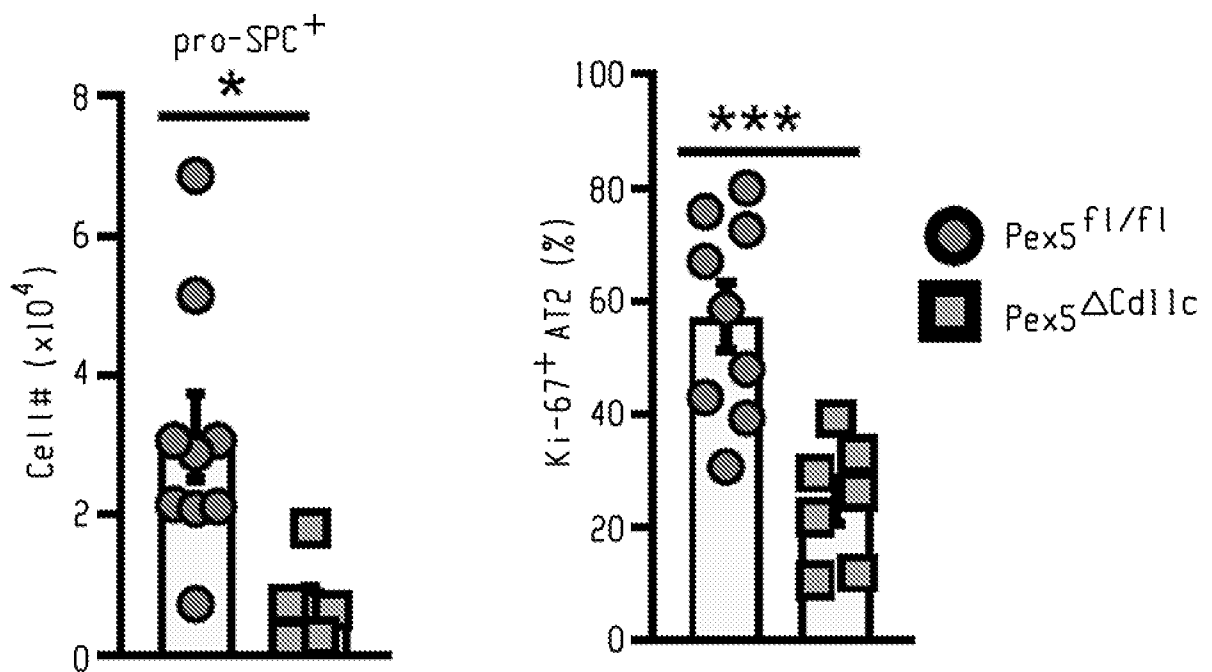
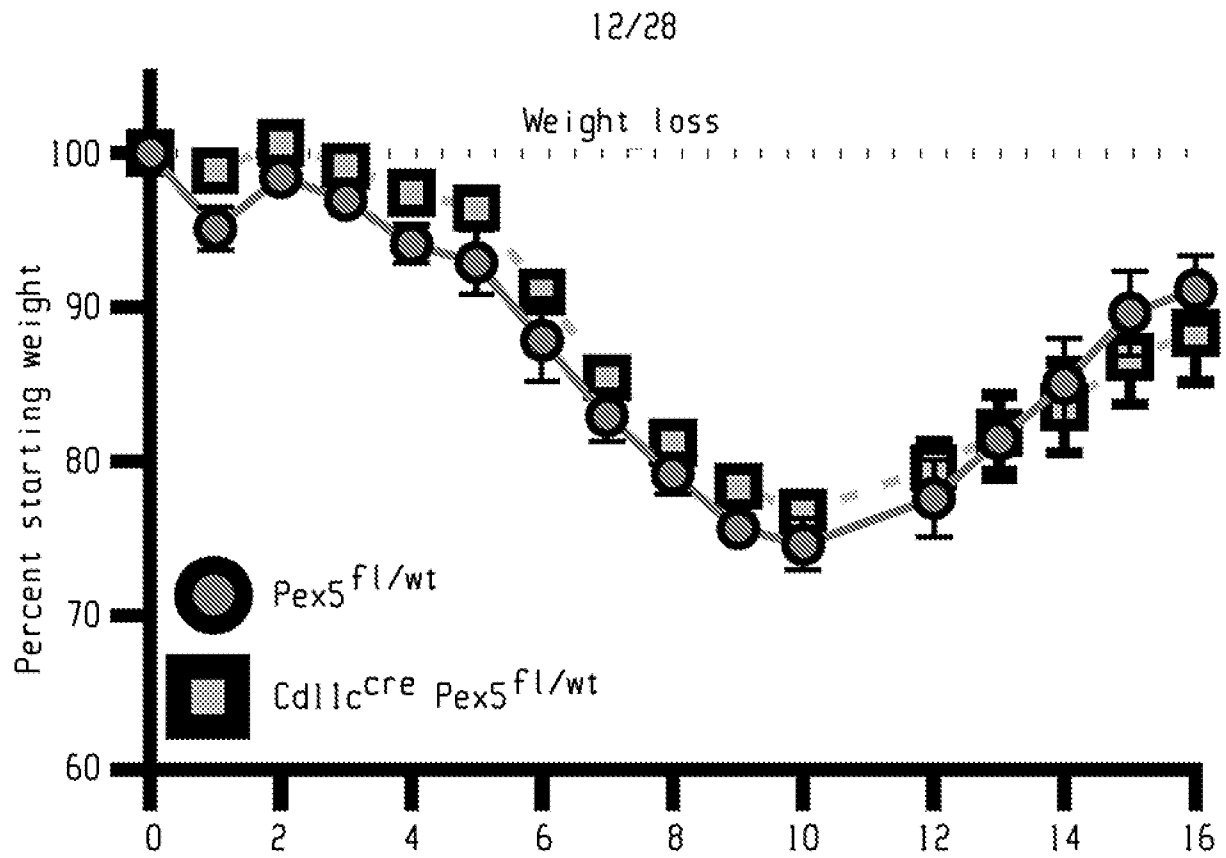
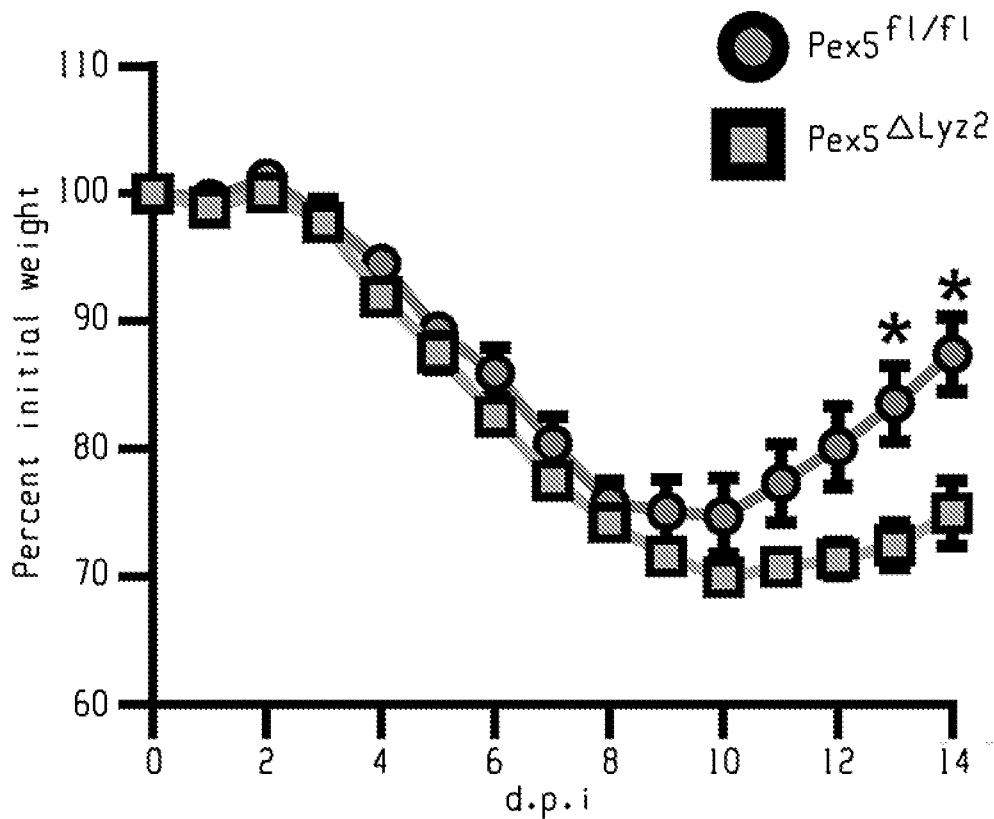
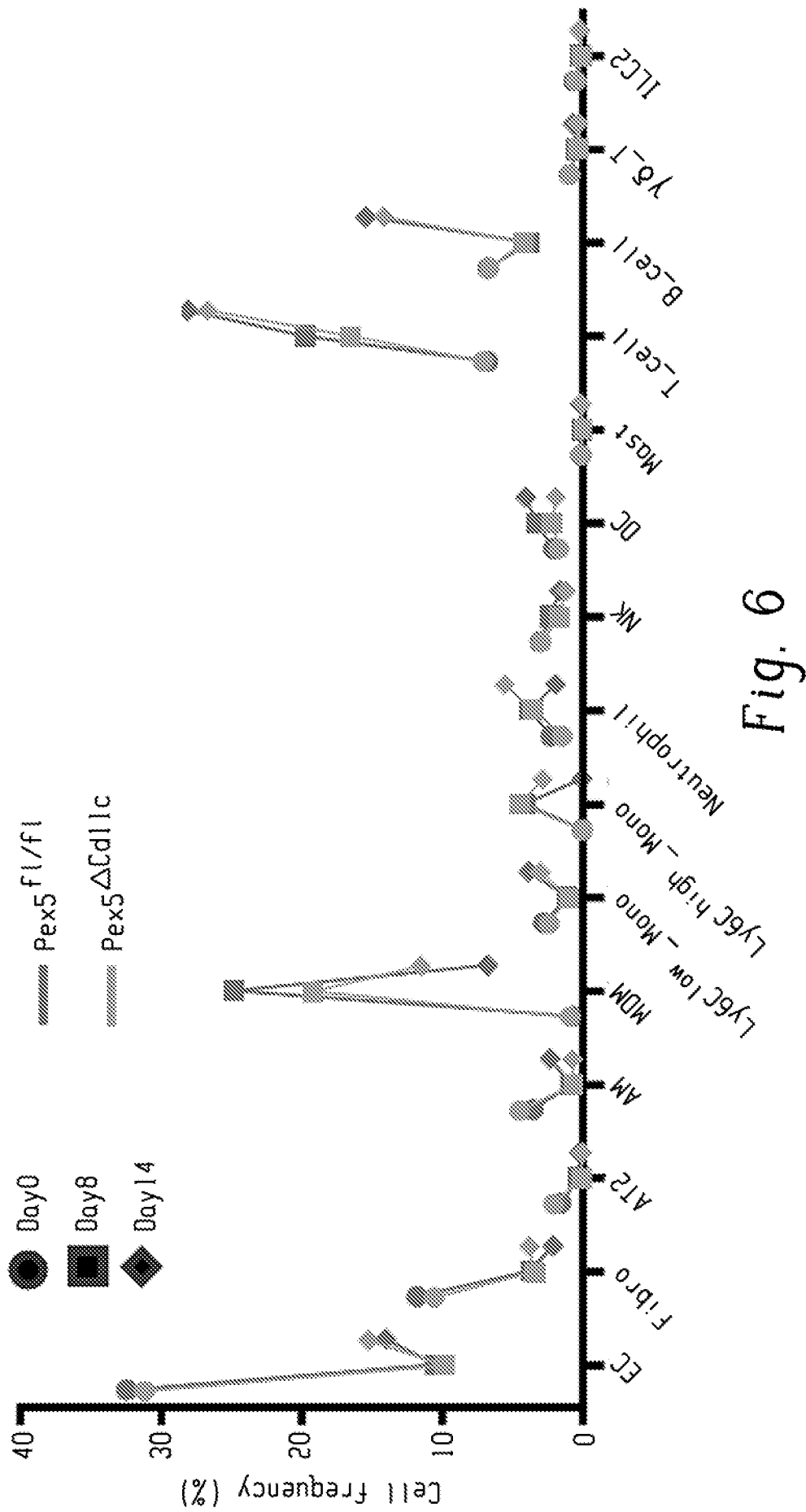


Fig. 4H

*Fig. 4I**Fig. 4J*

*Fig. 5A**Fig. 5B*

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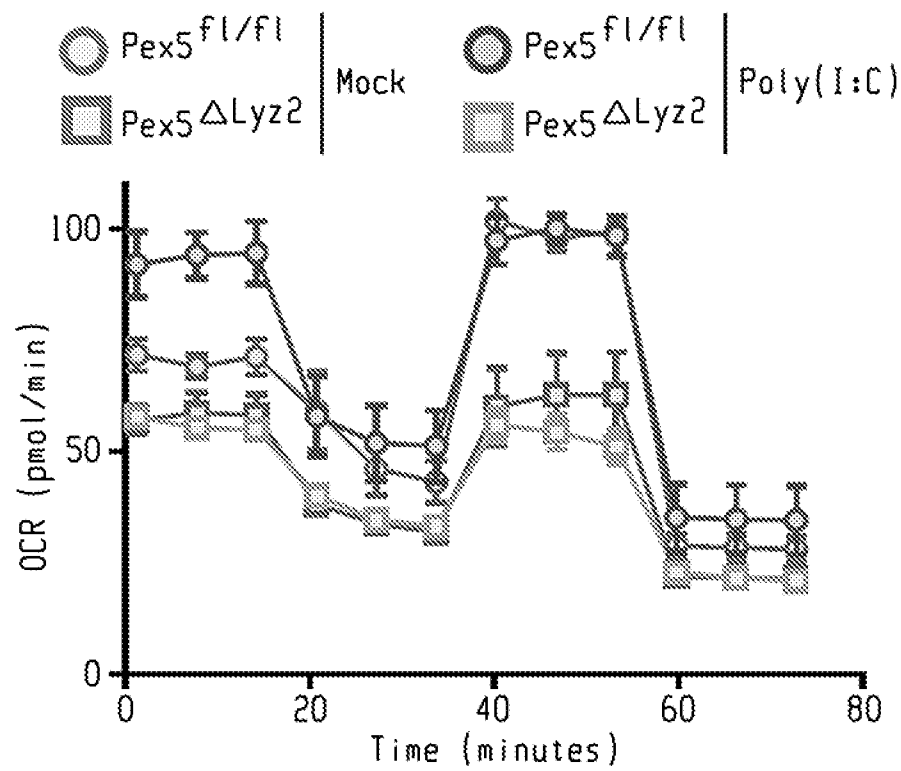


Fig. 7A

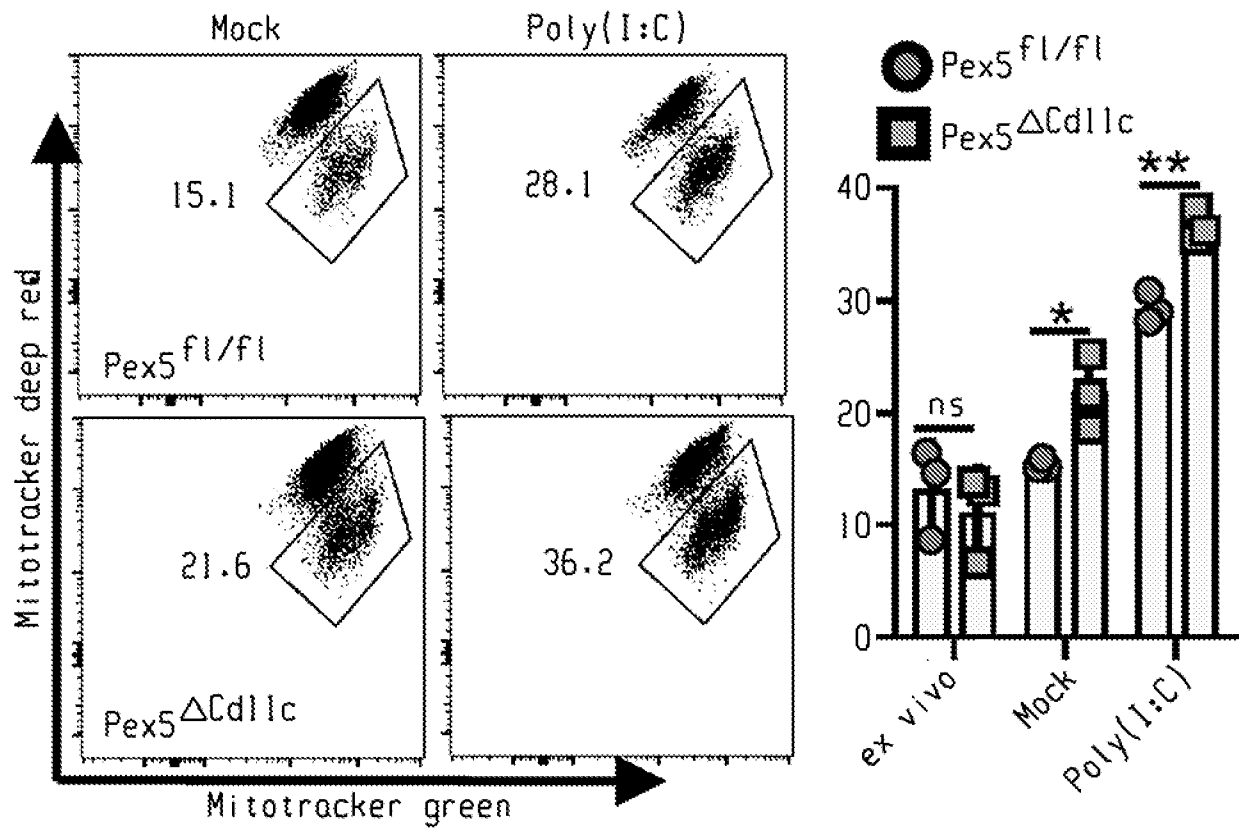


Fig. 7B

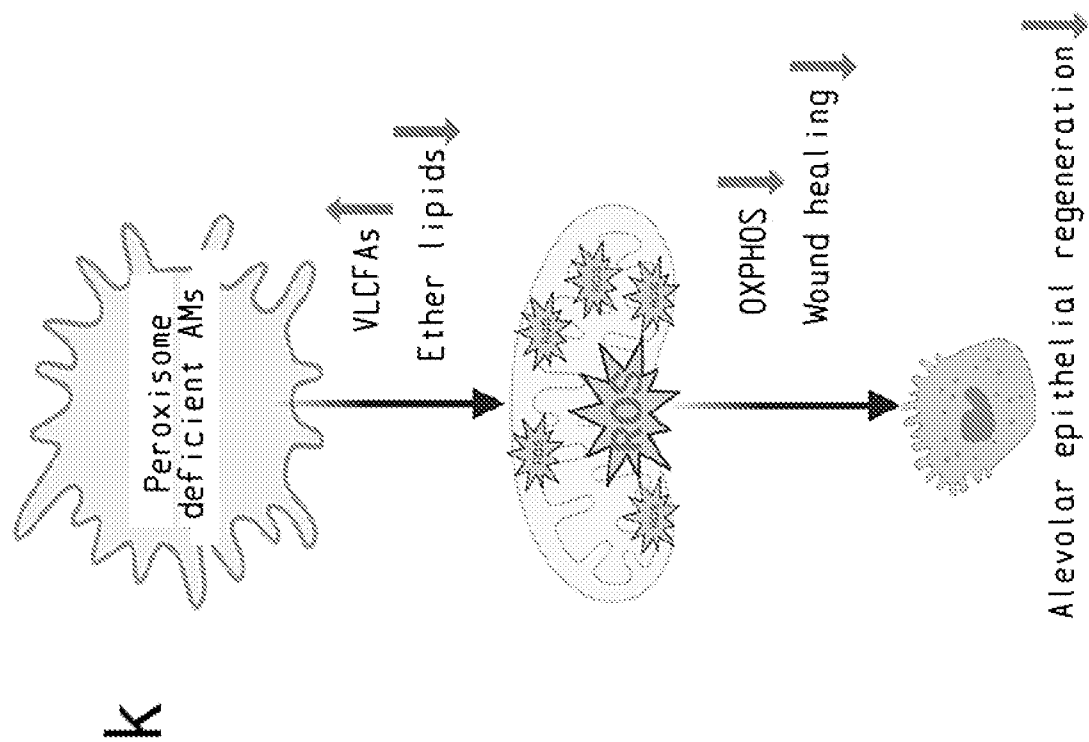


Fig. 7D

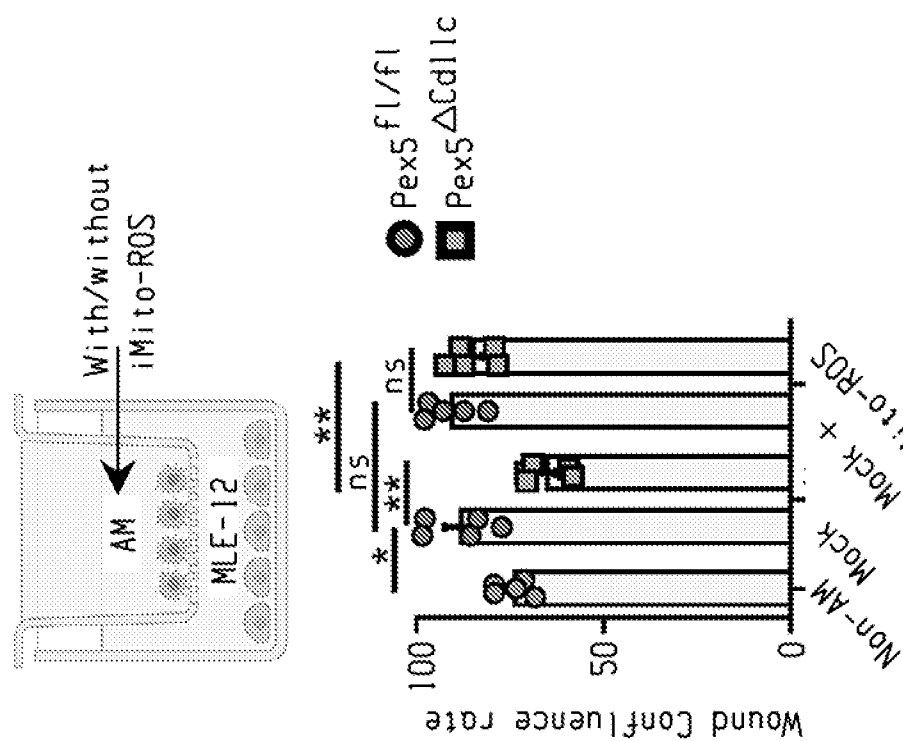
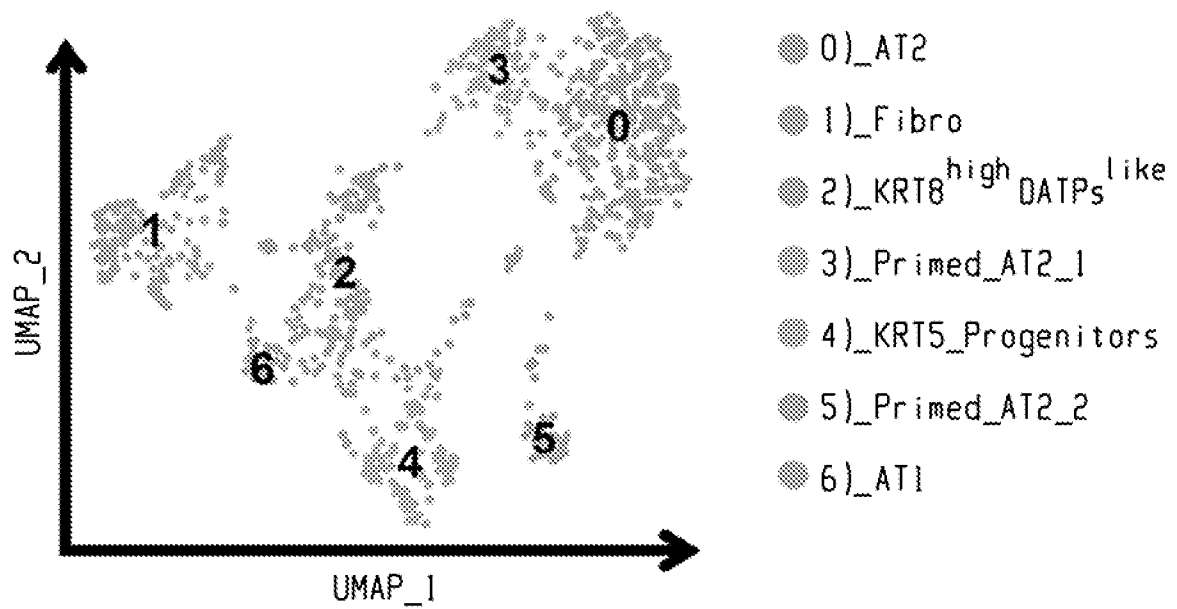
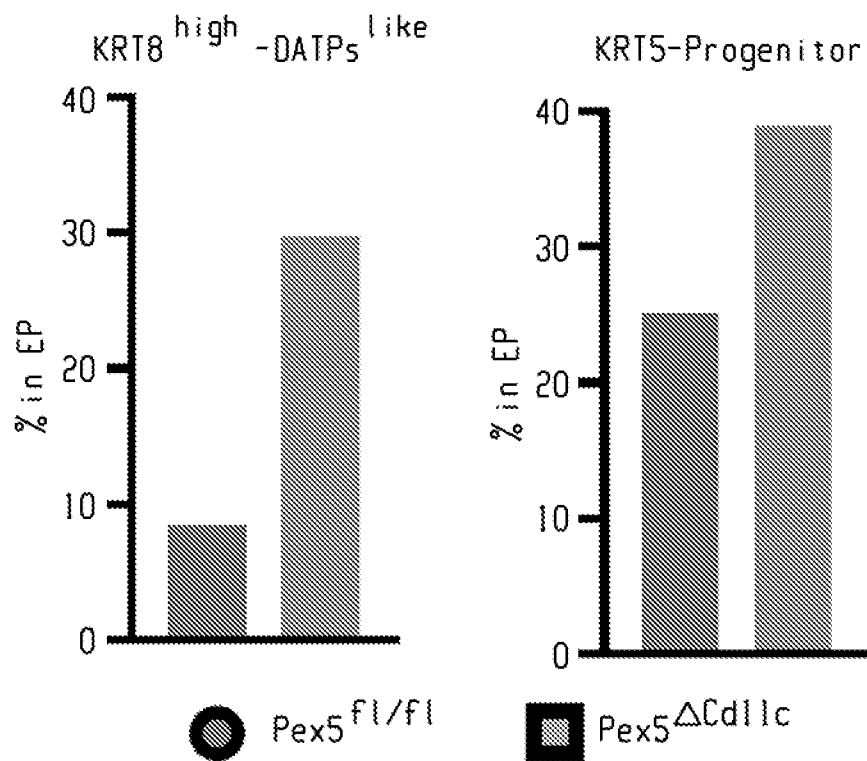


Fig. 7C

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*Fig. 8A**Fig. 8B*

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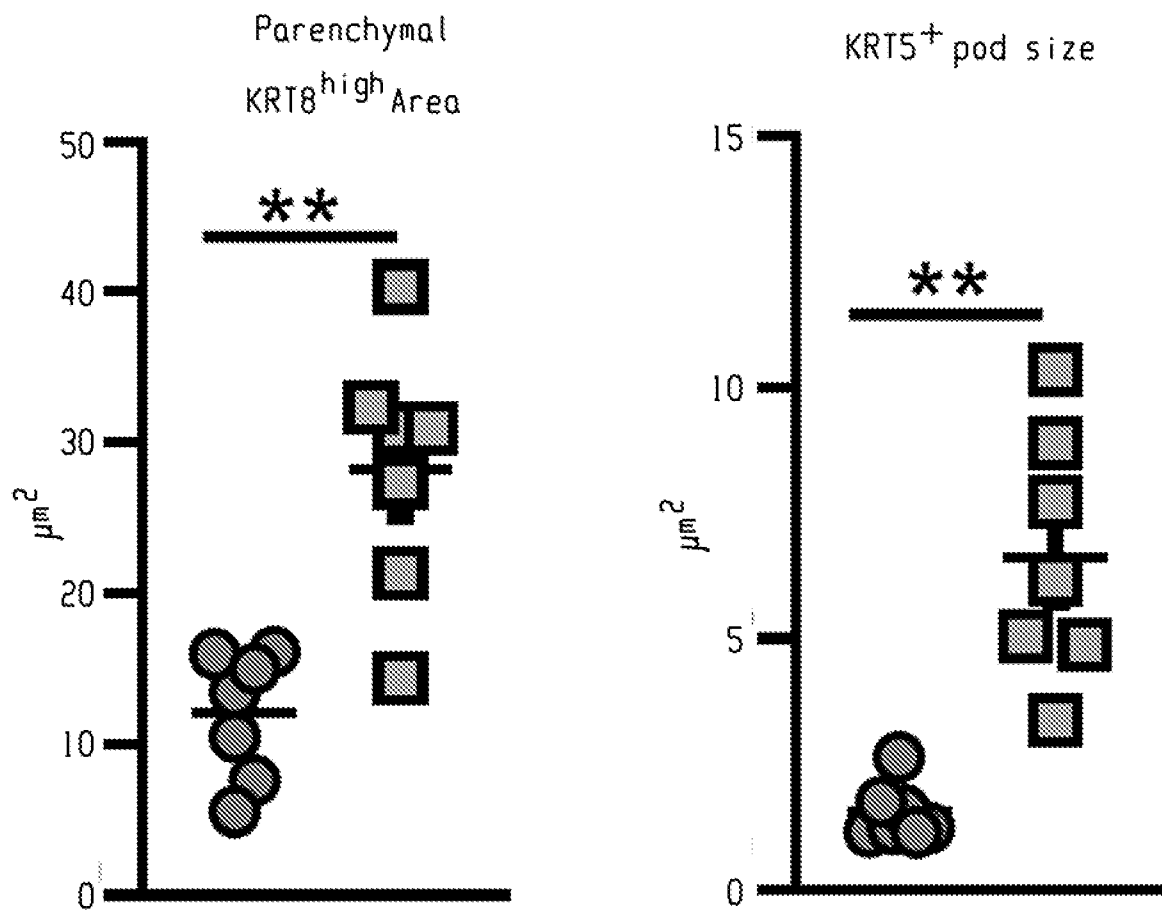


Fig. 8C

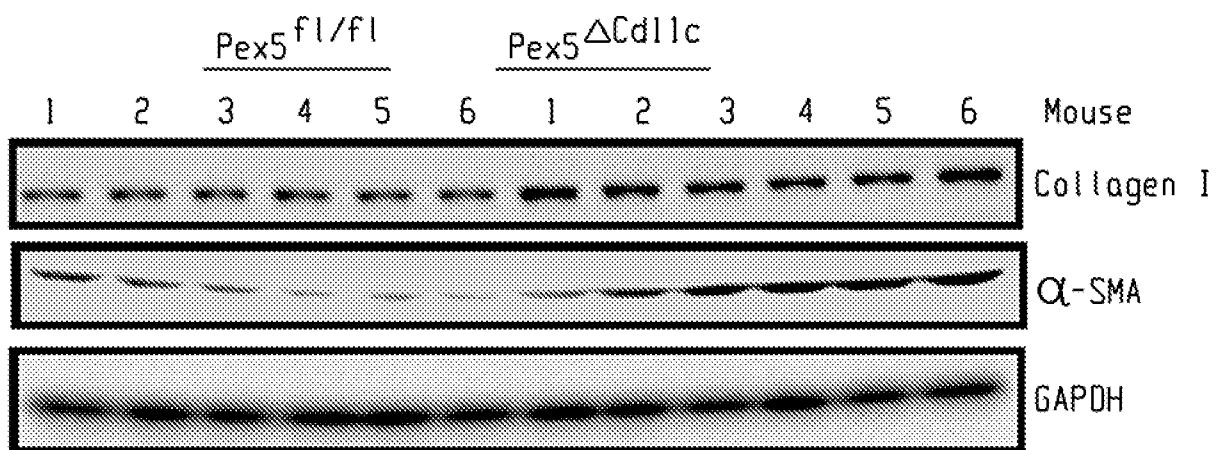
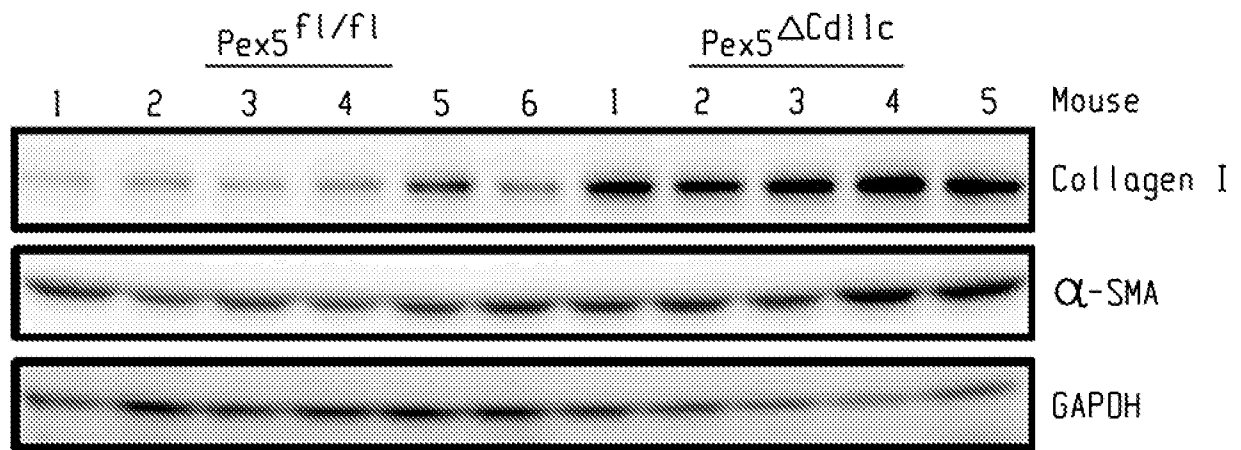
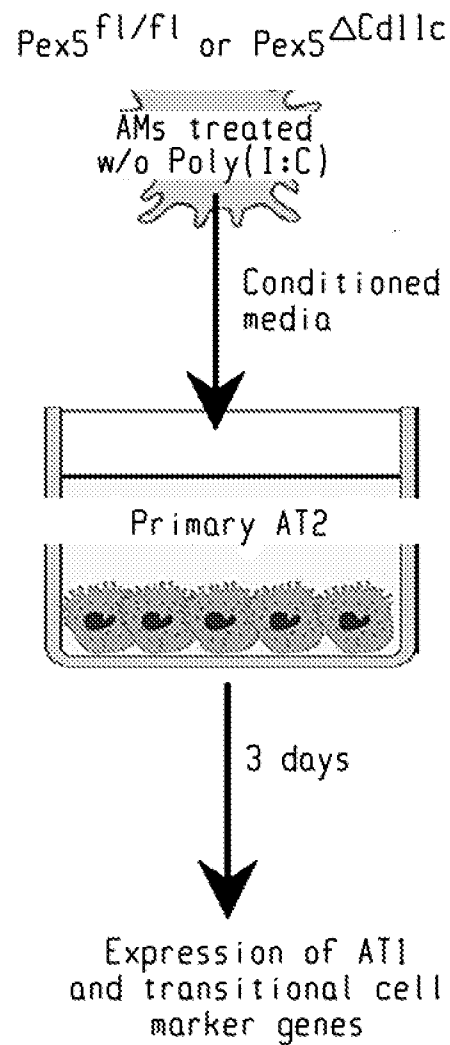
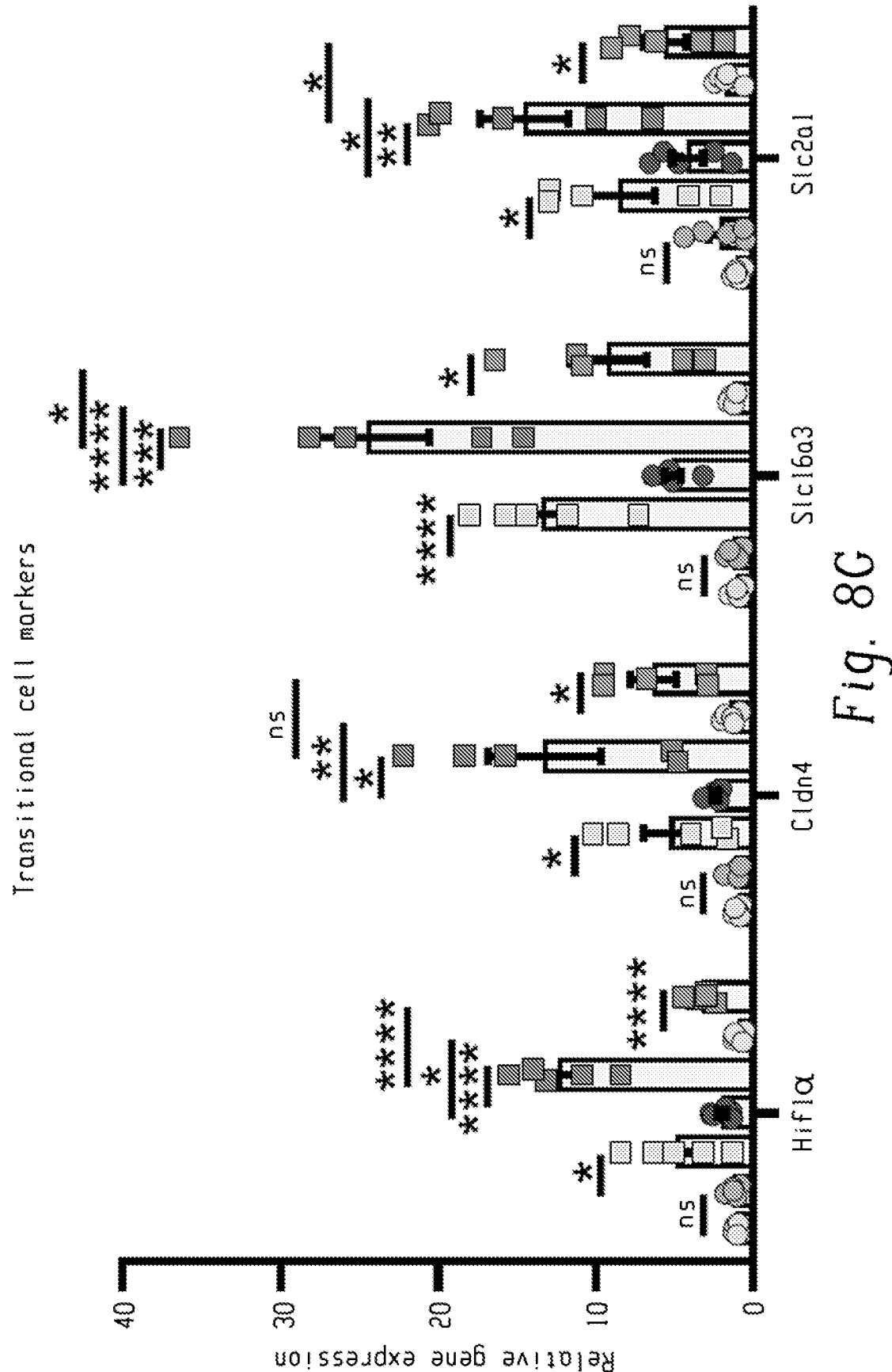


Fig. 8D

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*Fig. 8E**Fig. 8F*



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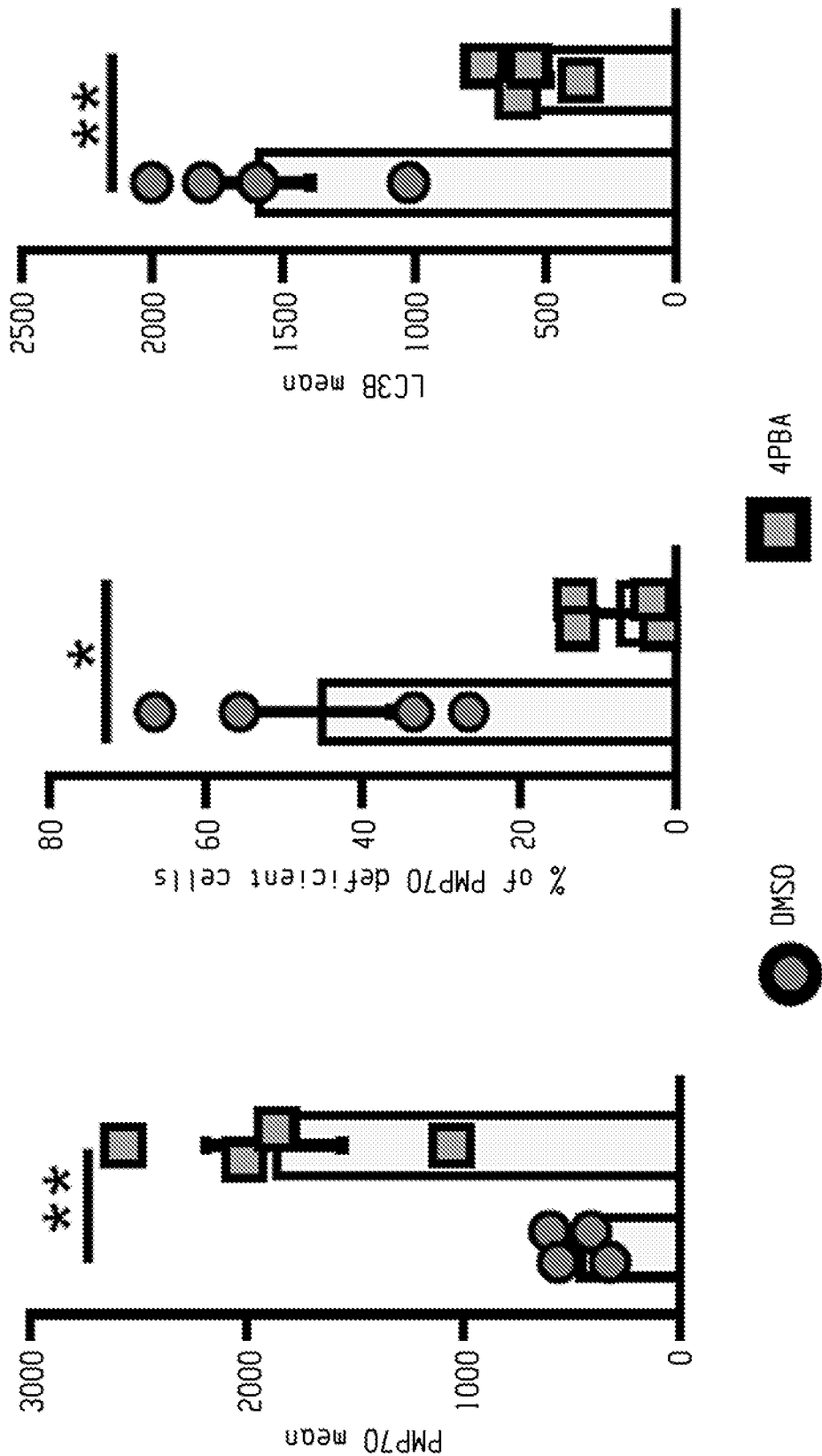
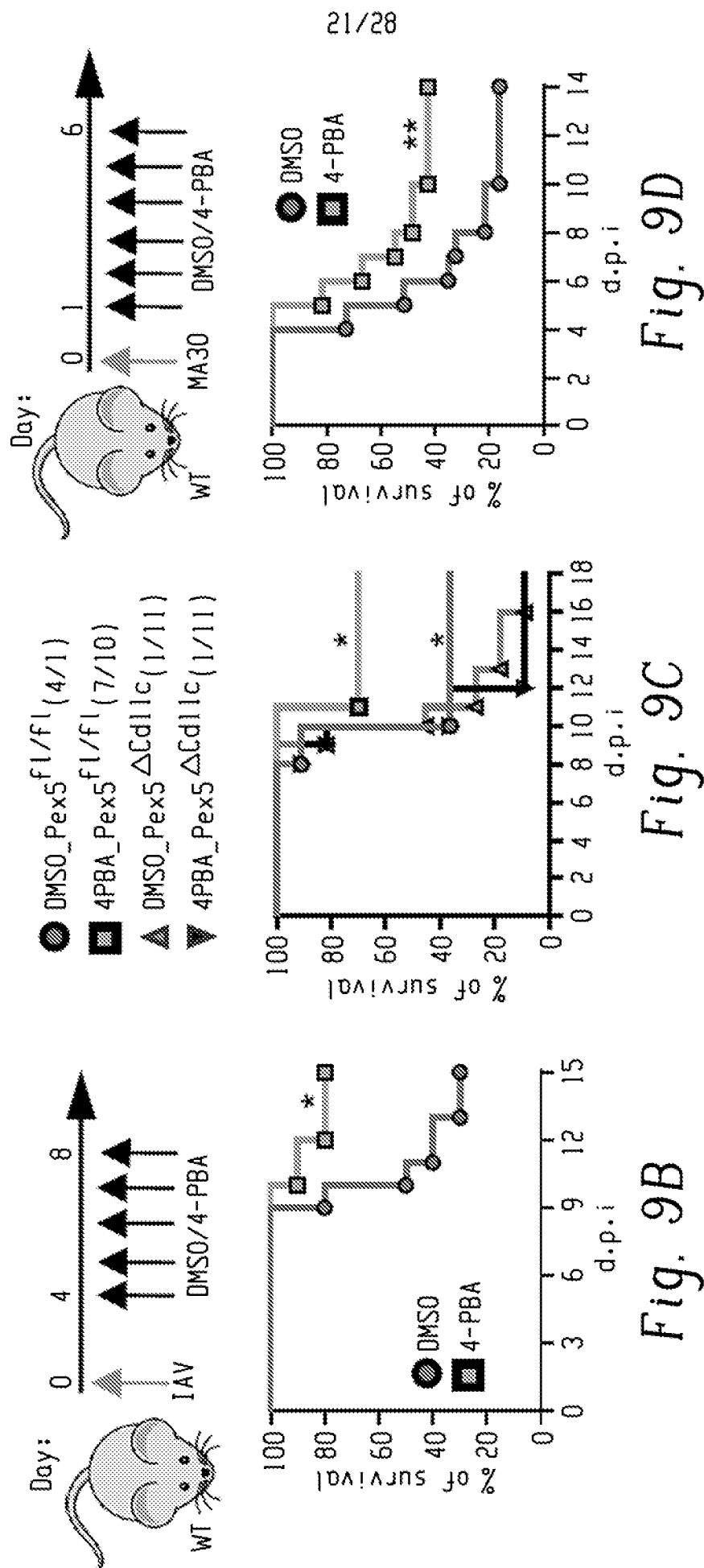


Fig. 9A



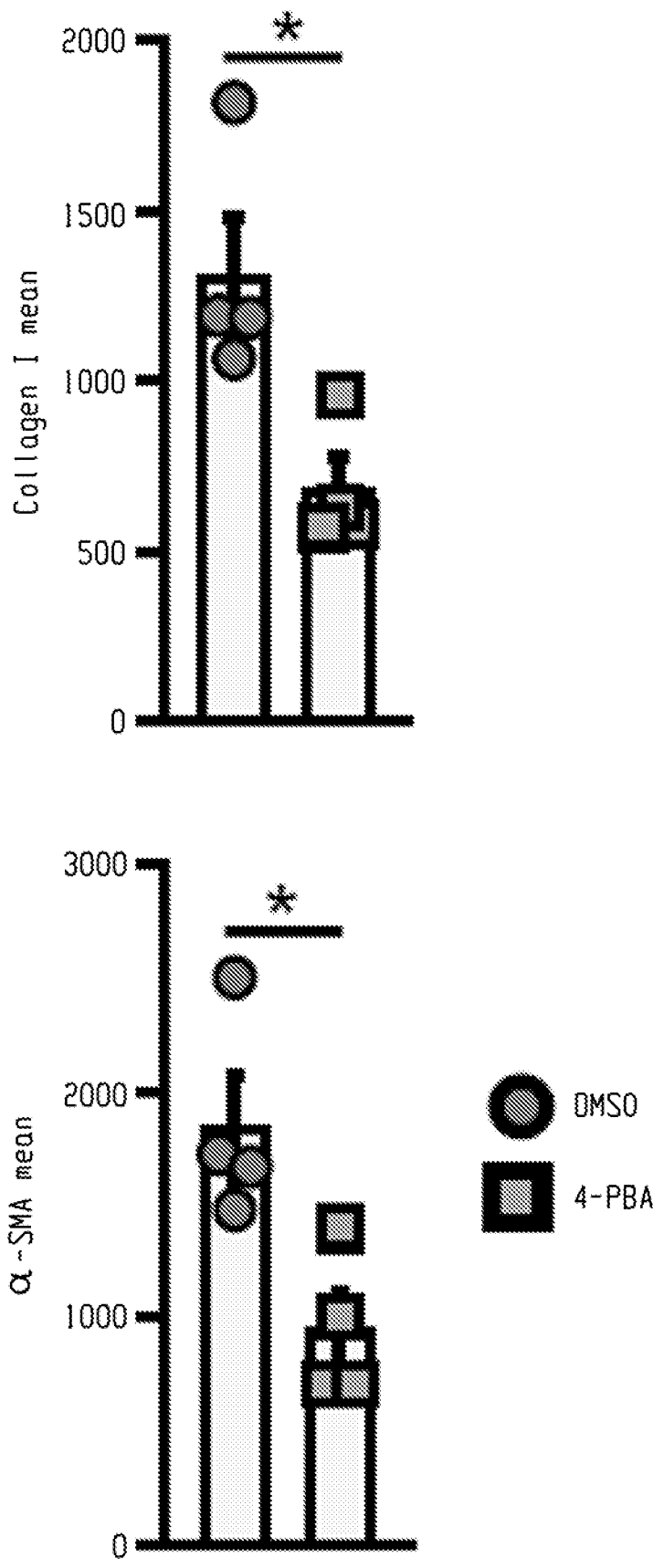


Fig. 9E

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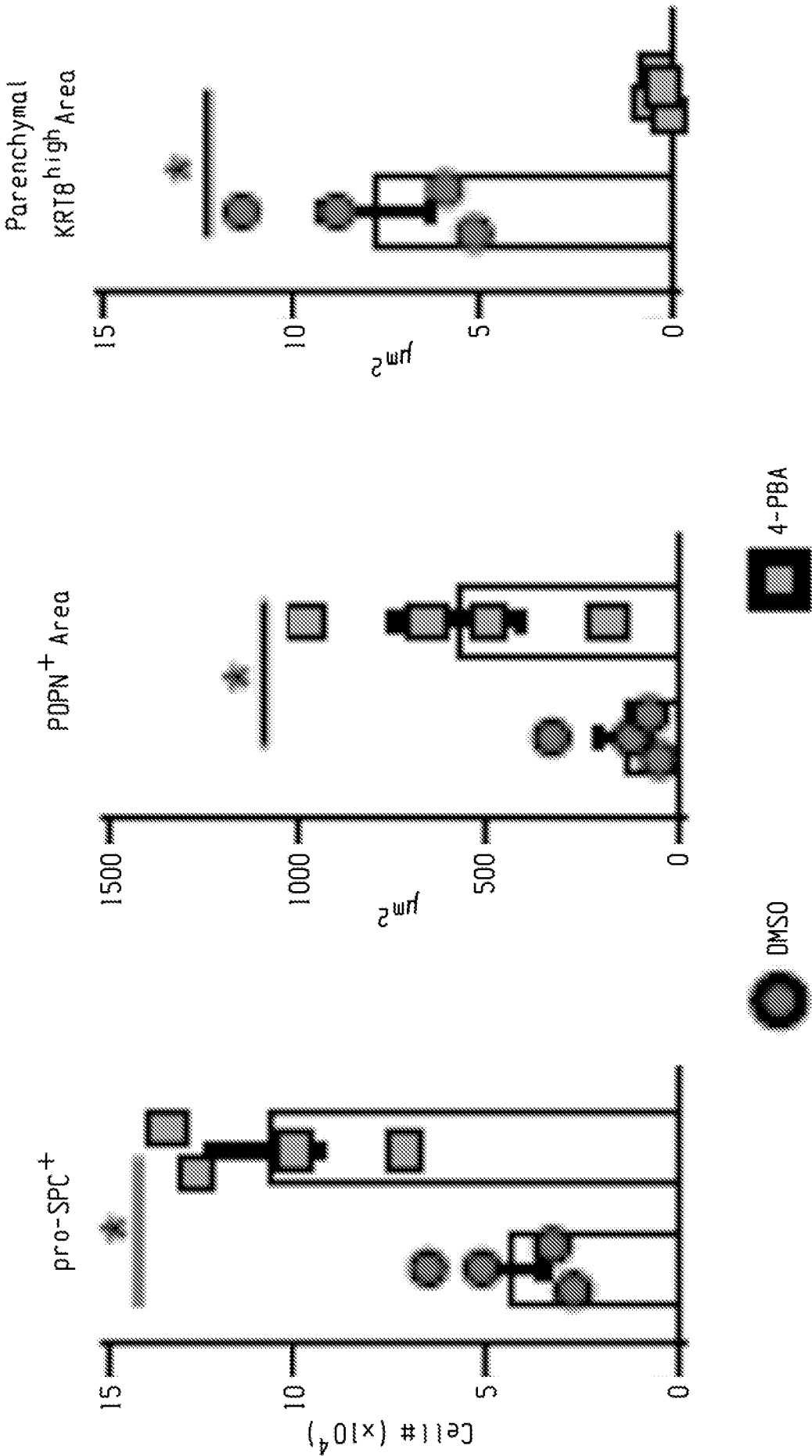
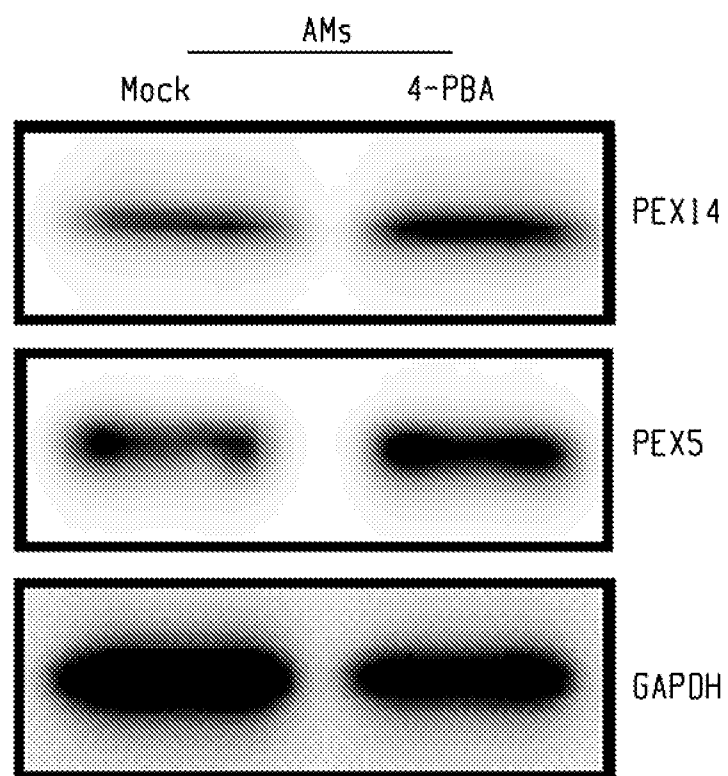
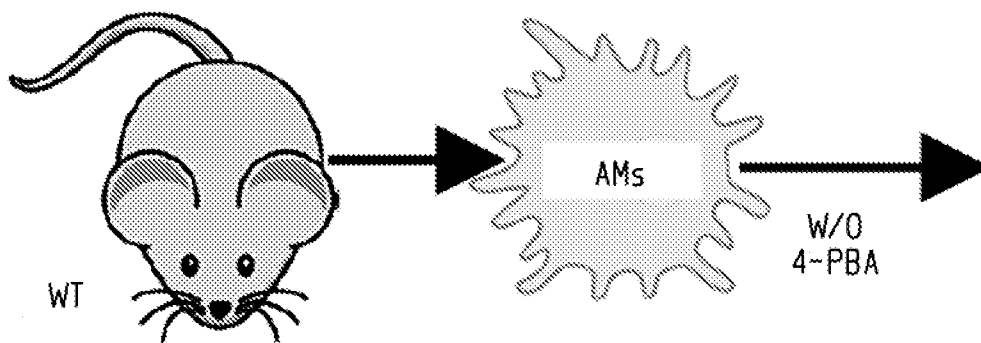
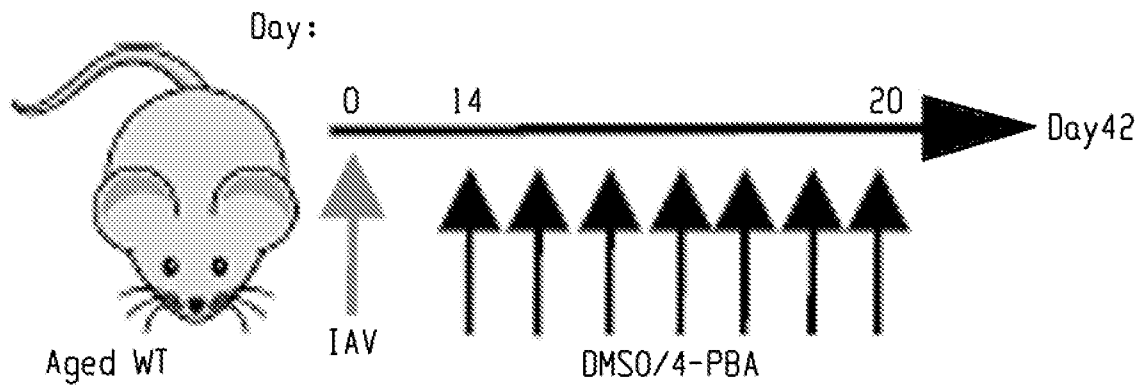
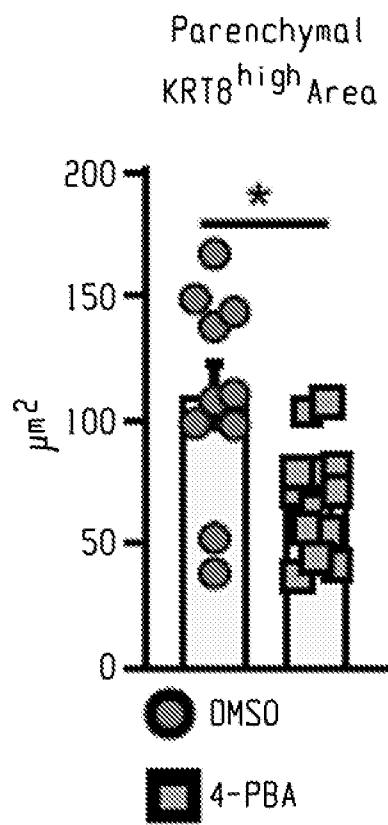
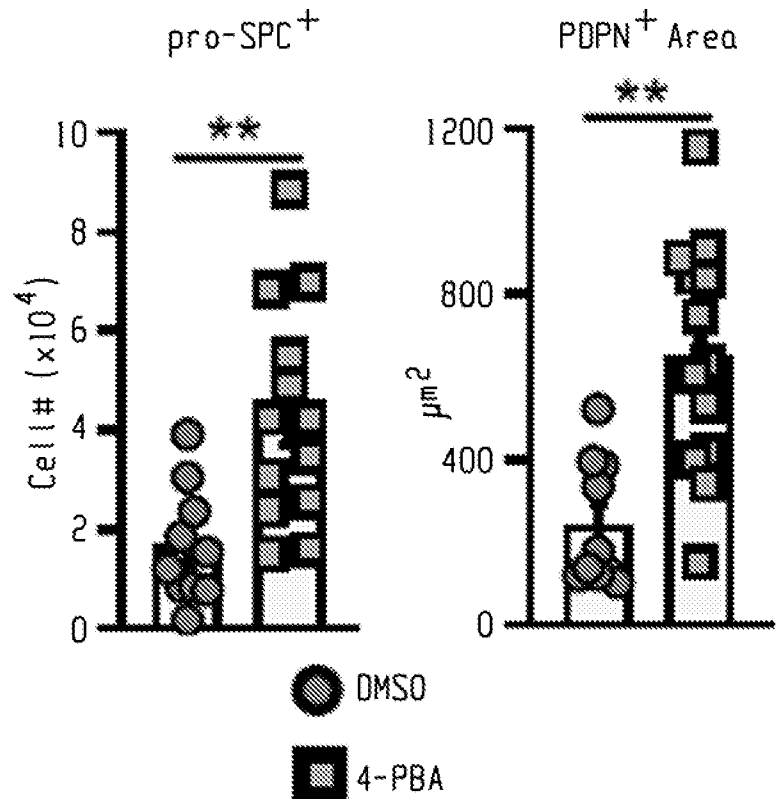


Fig. 9F

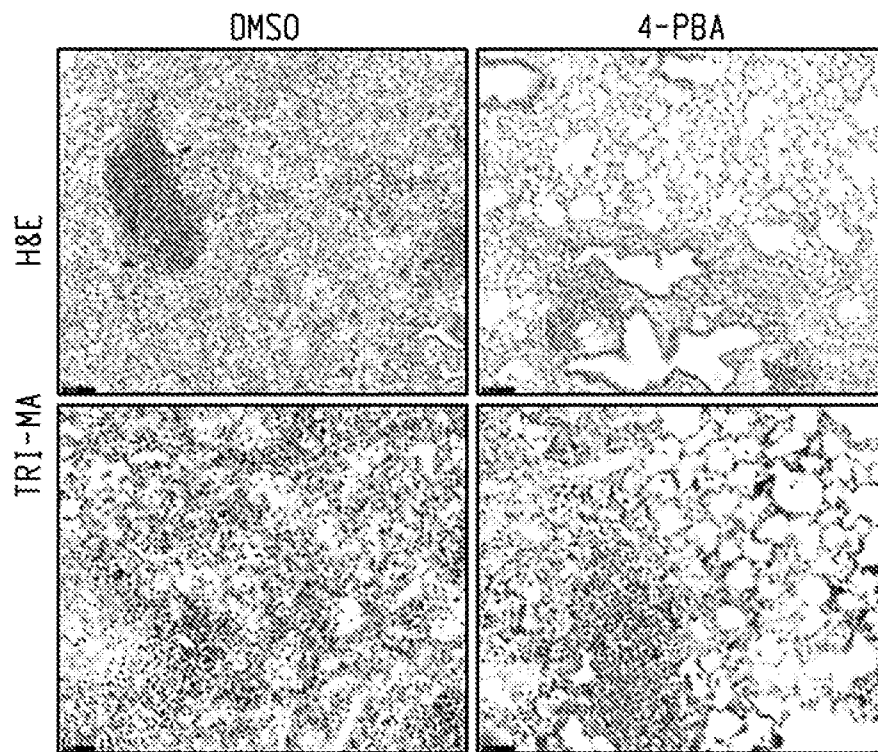
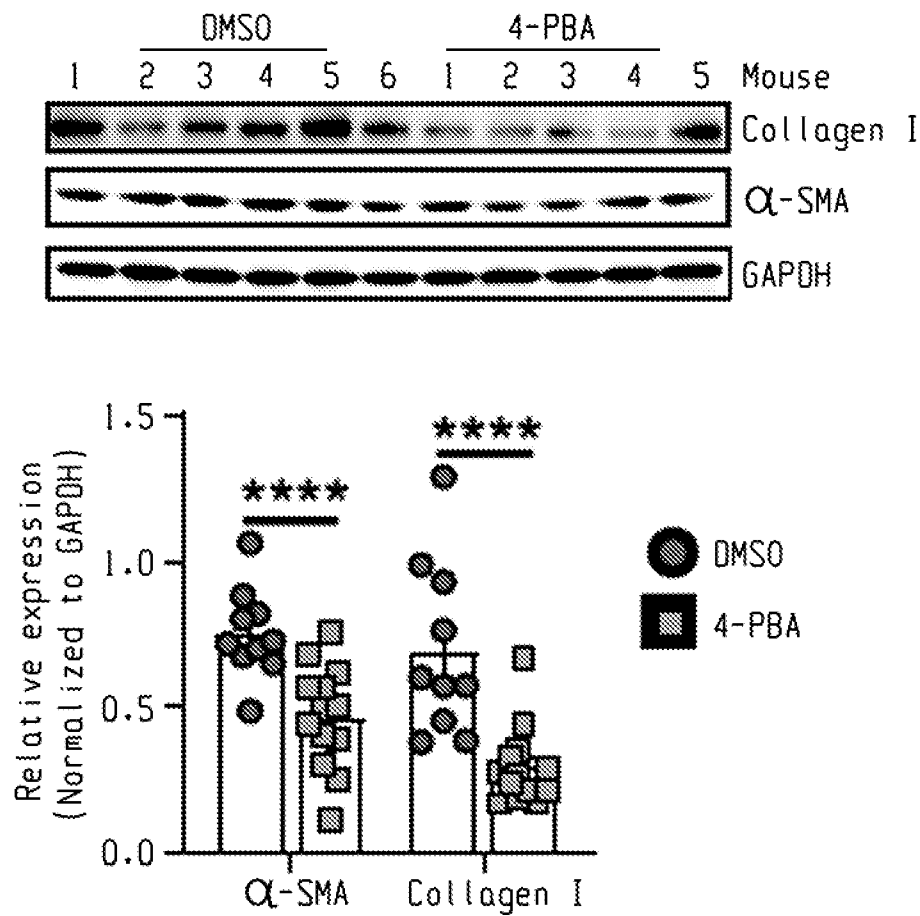
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*Fig. 10*

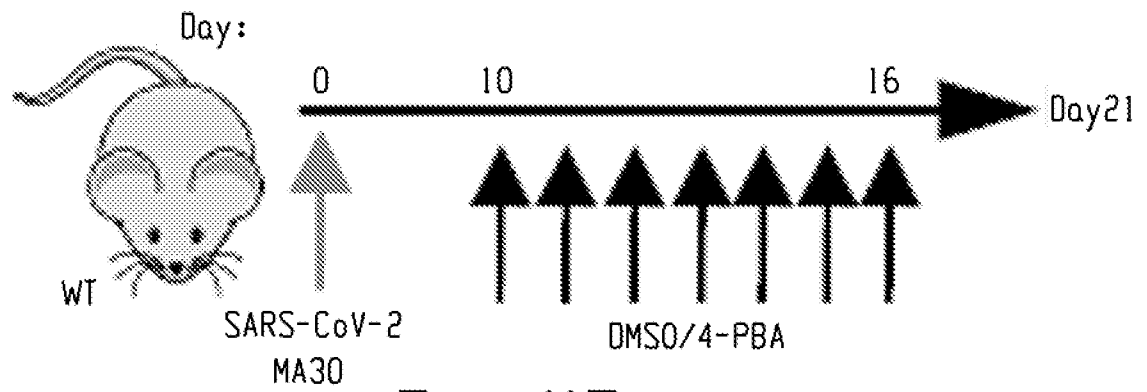
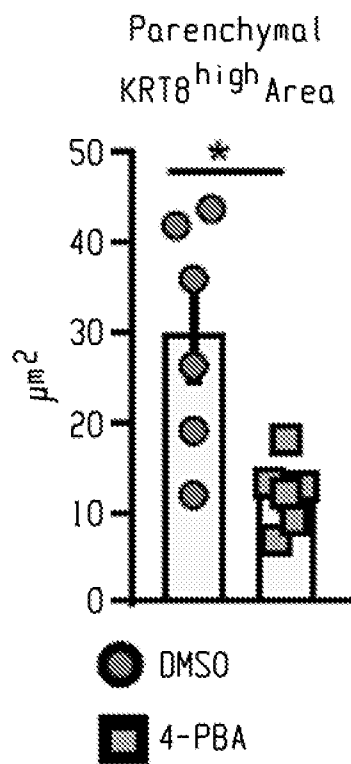
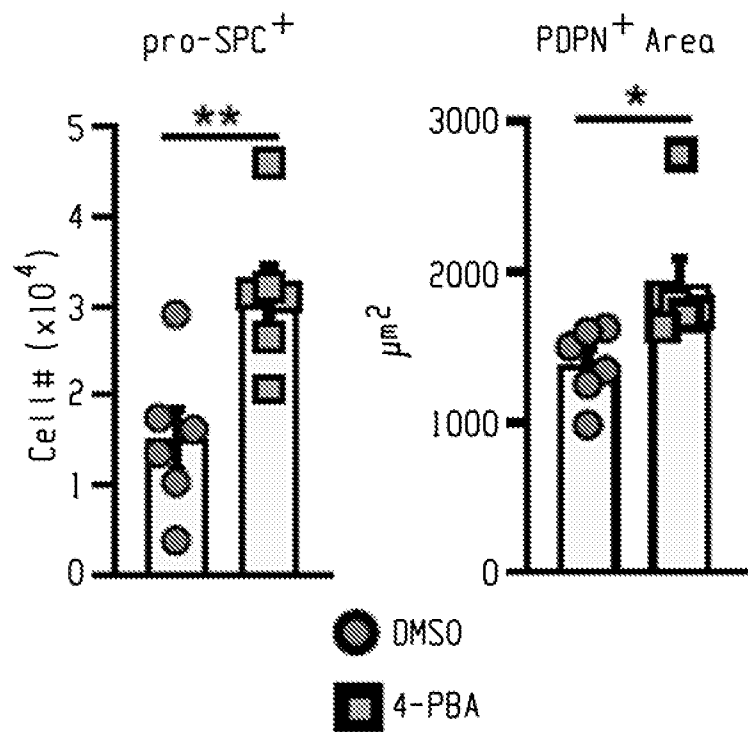
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*Fig. 11A**Fig. 11B**Fig. 11C*

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*Fig. 11D**Fig. 11E*

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*Fig. 11F**Fig. 11G**Fig. 11H*

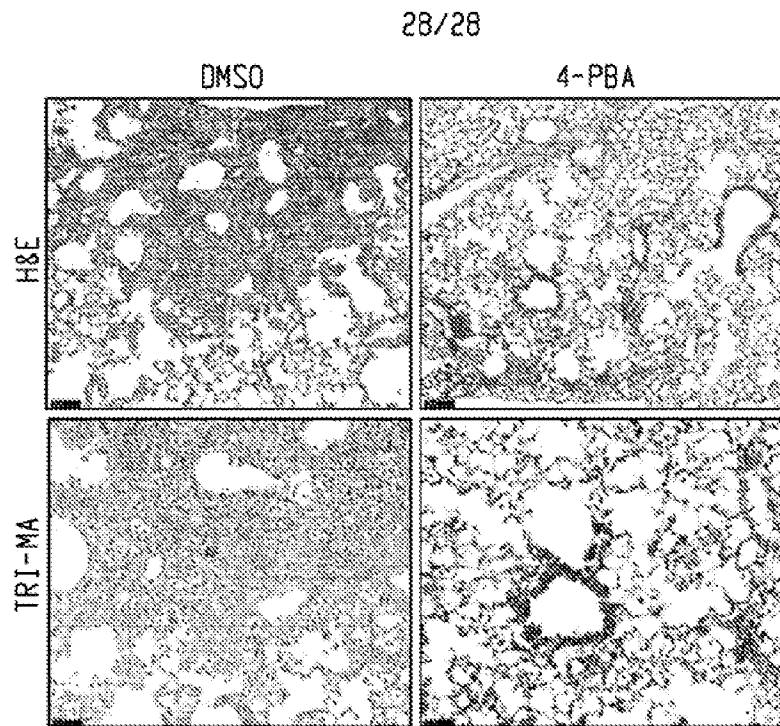


Fig. 11I

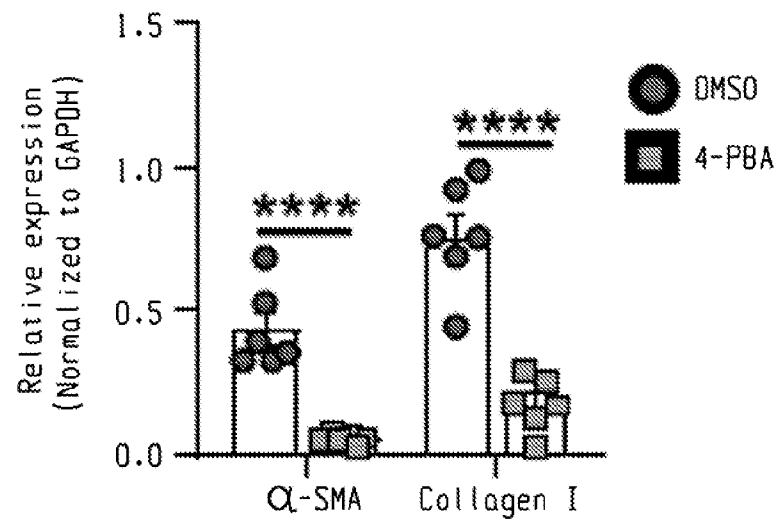
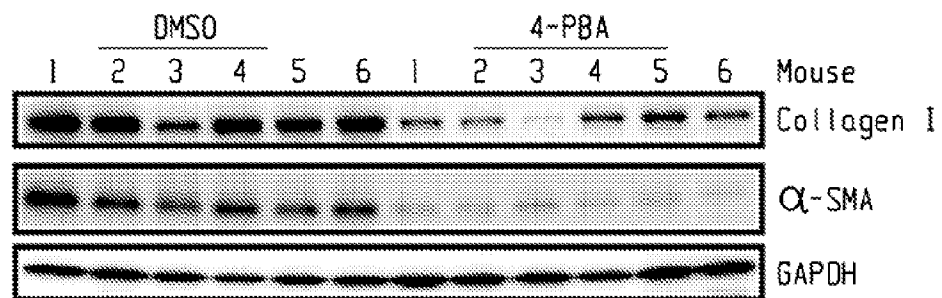


Fig. 11J

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/051510

A. CLASSIFICATION OF SUBJECT MATTERIPC: **A61K 31/192** (2023.01); **A61P 11/06** (2023.01); **A61P 29/00** (2023.01); **A61P 31/12** (2023.01)CPC: **A61K31/192; A61P11/06; A61P29/00; A61P31/12; A61K31/202; A61P3/00; A61P25/00**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,552,081 B1 (FREEDMAN, S. et al.) 22 April 2003 (22.04.2003) column 12, lines 14-16; column 12, lines 14-16, and lines 37-42	1, 9-10, 11/9, 12/11/9
Y	US 2022/0031700 A1 (MELIOR PHARMA I INC) 03 February 2022 (03.02.2022) claims 1, 26; paragraph [0176]	4-6, 11/4-6, 12/11/4-6
X	HASANKHANI. "The role of peroxisome proliferator-activated receptors in the modulation of hyperinflammation induced by SARS-CoV-2 infection: A perspective for COVID-19 therapy" Frontiers in Immunology. Web. 17 February 2023; DOI: 10.3389/fimmu.2023.1127358 page 2, column 1, paragraph 2; page 3, column 2, paragraph 1; page 9, column 2, paragraph 3; page 3, column 2, paragraph 2; page 10, column 1, paragraph 1	1-3, 7, 11/1-3, 11/7, 12/11/1-3, 12/11/7
Y		4-6, 11/4-6, 12/11/4-6
X	DOMINGUEZ. "A High Docosahexaenoic Acid Diet Alters the Lung Inflammatory Response to Acute Dust Exposure" Nutrients. Web. 04 August 2020; DOI: 10.3390/nu12082334 Abstract	1, 8, 11/8, 12/11/8

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

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Date of the actual completion of the international search

11 December 2024 (11.12.2024)

Date of mailing of the international search report

23 December 2024 (23.12.2024)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/051510

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEE. "Harnessing peroxisome proliferator-activated receptor γ agonists to induce Heme Oxygenase-1: a promising approach for pulmonary inflammatory disorders" Cell Communication and Signaling. Web. 15 February 2024; DOI: 10.1186/s12964-024-01501-4 page 2, column 2, paragraph 1; page 11, column 1, paragraph 1; page 3, column 1, paragraph 2; page 7, column 2, paragraph 6; page 9, column 2, paragraph 3	1, 4, 8, 11, 12
P,Y	SAHA. "Peer reviewed Academic Journal Tools for Idiopathic pulmonary fibrosis (IPF): disease pathophysiology, targets, and potential therapeutic interventions. Idiopathic pulmonary fibrosis (IPF): disease pathophysiology, targets, and potential therapeutic interventions." 2181–2194. Molecular & Cellular Biochemistry. Web. October 2024; DOI: 10.1007/s11010-023-04845-6 page 2182, column 1, paragraph 2; page 2184, column 1, paragraph 3	8